Detection of bacterial gene expression by a novel isothermic nucleic acid amplification technology

Thomas, Alistair Owen

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Detection of bacterial gene expression by a novel isothermic nucleic acid amplification technology

Submitted by Alastair Owen Thomas
For the degree of Doctor of Philosophy
University of Bath
Department of Pharmacy and Pharmacology

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

A novel probe-based technique called Signal-Mediated Amplification Reaction Technology (SMART) was optimised for detection of RNA targets in order to quantify gene expression. The SMART assay was used to quantify both 23S rRNA in *P. aeruginosa* PAO1 and *gfp*mut3 mRNA in the plasmid-borne *rpoS*::*gfp*mut3 fusions *P. aeruginosa* SS429 and SS431. However, the assay was not sufficiently sensitive to detect *gfp*mut3 mRNA from the chromosomal *rpoS*::*gfp*mut3 fusion *P. aeruginosa* SS336.

SDS-PAGE analysis of outer membrane proteins of *P. aeruginosa* PAO1 revealed that cells grown in a reported iron-replete chemically defined medium CDM_{10} were in fact limiting for iron. Modifications to the growth medium such as increased iron concentration, reduction in pH and the addition of citrate and ascorbate all failed to produce an iron-replete phenotype. This was achievable only when MOPSO buffer was replaced with phosphate buffer, indicating that by some unknown mechanism MOPSO can reduce iron availability in minimal media.

The effects of nutrient limitation on *rpoS* expression in *P. aeruginosa* planktonic and biofilm culture were investigated using direct fluorescence measurement of *rpoS*::*gfp*mut3 chromosomal and plasmid fusions. In planktonic culture, nutrient-replete and magnesium-limited conditions resulted in an increase in *rpoS* expression whilst minimal levels of *rpoS* expression were seen in both iron-limited and glucose-limited conditions. Furthermore, minimal expression of *rpoS* was noted in *P. aeruginosa* biofilms in glucose, magnesium and iron-limited conditions.

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ABBREVIATIONS

3WJ	three-way junction
AI	autoinducer
AMP	adenine monophosphate
AP	alkaline phosphatase
ATP	adenine triphosphate
°C	degrees centigrade
CDM	chemically defined medium
CF	cystic fibrosis
cfu	colony forming units
cm, mm, μm, nm	centimetre, millimetre, micrometre, nanometre
ddH_2O	double distilled water
ELOSA	enzyme-linked oligosorbent assay
FACS	fluorescence activated cell sorting
FMN	flavin mononucleotide
fu	fluorescence units
FUR	ferric uptake regulator
GFP	green fluorescent protein
h	hours
IROMPs	iron regulated outer membrane proteins
KDa	kilodaltons
l, ml,μl	litres, millilitre, microlitre
LB	Luria-Bertani broth
M, mM, μM,	molar, millimolar, micromolar
min	minutes
MOPS	3-(*N*-morpholino)propane sulphate
MOPSO	3-(*N*-morpholino)-2-hydroxy-propanesulfonic acid
MW	molecular weight
OD	optical density
ORF	open reading frame
PAI-1	*N*-(3-oxododecanoyl)-*L*-homoserine lactone

PAI-2	*N*-butyryl-*L*-homoserine lactone
PCR	polymerase chain reaction
poly P	inorganic polyphosphate
PPK	polyphosphate kinase
PQS	*Pseudomonas* quinolone signal
QS	quorum sensing
RNA-1	primary RNA signal
RNA-2	secondary RNA signal
RNA-3	tertiary RNA signal
RBS	ribosomal binding site
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMART	signal mediated amplification reaction technology
T7 RNAP	T7 RNA polymerase
TE	Tris-EDTA
g	times gravitational force
v/v	volume per unit volume

1 INTRODUCTION

1.1 *P. aeruginosa* AND INFECTION

P. aeruginosa is a ubiquitous Gram-negative bacterium found in a wide range of environmental niches including soil, water and plants (Rashid *et al.* 2000b) which reflects the organisms remarkable degree of physiological and genetic adaptability (Spiers *et al.* 2000). *Pseudomonas* spp. also demonstrate broad metabolic versatility, degrading substrates including aromatics, halogenated derivatives and many other organic compounds (Stanier *et al.* 1966). *P. aeruginosa* is an opportunistic pathogen that can infect immunocompromised patients with conditions such as cancer, leukemia, HIV infections, heart disease, diabetes and severe burns and wounds (Wick *et al.* 1990). It is also the primary cause of morbidity and mortality in cystic fibrosis patients (Govan and Deretic 1996). In order colonise a variety of environments *Pseudomonas* spp. secrete a variety of extracellular factors to acquire nutrients and evade host defences which maintain the viability and growth of the population. The production of these factors is regulated by an elaborate network of response regulators that are used to sense population size and environmental stresses such as starvation, pH, desiccation and temperature. *P. aeruginosa* secretions are responsible for it pathogenesis in humans especially in disorders such as cystic fibrosis (CF) in which the iron starved conditions of the lung induce the expression of pyoverdin and exotoxin A which have both been shown to induce heavy tissue damage via the formation of immune complexes (Cochrane 1988). Therefore the investigation of the expression of genes that regulate virulence determinants under various stresses would reveal how the bacteria respond to conditions that would be commonly encountered in the host and perhaps identify factors that could be implemented to reduce the pathogenicity of *P. aeruginosa* in disorders such as cystic fibrosis.

1.2 THE GENERAL STRESS RESPONSE

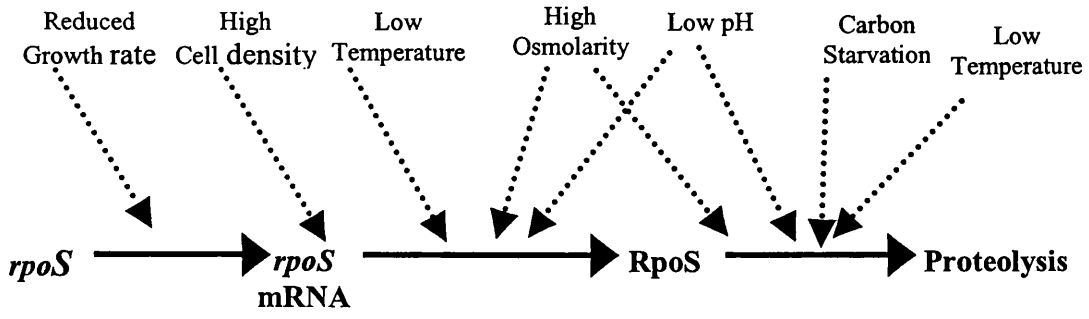
The general stress response is characterised by numerous alterations in cellular physiology and morphology that enhance survival by increasing cellular stress resistance, which prevents cellular damage rather than repairing it (Hengge-Aronis 2000). Unlike sporalating bacteria, which demonstrate a reactive, all-or-nothing approach to dormancy, the stationary phase of Gram-negative bacteria is a reversible state from which they can return to exponential growth when the stress is diminished. This flexibility is conducive to the colonisation of a wide selection of environments and allows the bacteria to respond in a stress-specific manner. Although the phenotype expressed by the bacterium in response to stress is the cumulative expression of a wide selection of genes, the coordination of the stress response is commonly regulated by the stationary phase sigma factor RpoS, also termed σ^s or σ^{38}.

1.2.1 RpoS and *E. coli*

Initial research into RpoS was conducted in *E. coli* by several groups who discovered a gene which regulated catalase and acid phosphatase production and exonuclease III In log-phase growth the specificity of the RNA polymerase core enzyme is directed by housekeeping sigma factors such as RpoD, RpoE and RpoF with minimum expression of *rpoS*. As cells enter stationary phase RpoS levels rapidly increase to almost 40% of RpoD levels, effectively competing for the core enzyme and directing transcription to stress-specific and stationary-phase genes (Jishage *et al.* 1996). The *rpoS* operon consists of *rpoS* and *nlpD* (which encodes a lipoprotein of unknown function). The *nlpD* promoters contribute substantially to the basal level of *rpoS* expression in exponentially growing cells (Lange and Hengge-Aronis 1994b) but growth-phase-regulated transcription is initiated at additional sites within the *nlpD* ORF (Lange *et al.* 1995). The regulation of RpoS activity in *E. coli* is a complex network of transcriptional, posttranscriptional and proteolytic control (illustrated in Fig 1.1) which consolidates its ability to respond to a variety of stresses (Hengge-Aronis 2000). However, mRNA turnover does not appear to be a major point of regulation, with relatively high levels of *rpoS* mRNA detected even in exponentially growing cells and levels do not seem to change in response to several stresses that

result in strongly elevated RpoS protein levels (Hengge-Aronis 2002). Evidence indicates that in *E. coli*, RpoS can be regulated at the post-transcriptional level via secondary structure-based interaction with ssRNAs (small sub-unit RNAs) such as DsrA (Repoila and Gottesman 2002) and RprA (Majdalani *et al.* 2002). However, the predominant regulation of RpoS activity is at the post-translational level by CLP-XP protease (Klauck *et al.* 2003). Constant proteolysis by CLP-XP protease during log-phase growth levels ensures RpoS levels are at a minimum (Lange and Hengge-Aronis 1994a). However, various stresses result in stabilisation and rapid accumulation of the RpoS protein and the onset of the general stress response. This is achieved through regulation of the recognition factor RssB, which is essential for RpoS degradation in log-phase growth but is rendered inactive by dephosphorylation in response to certain stress conditions (Pruteanu and Hengge-Aronis 2003) (Klauck *et al.* 2003).

Fig. 1.1 The effect of various environmental stresses of the transcriptional, translational and proteolytic control of RpoS adapted from Hengge-Aronis (2002)



1.2.2 RpoS and *P. aeruginosa*

Following the cloning of the *P. aeruginosa rpoS* gene (Tanaka and Takahashi 1994), its role as a regulator of the stress response has been confirmed by *rpoS* mutants demonstrating increased sensitivity to heat shock, osmotic stress and hydrogen peroxide (Suh *et al.* 1999). However, stationary-phase *rpoS* mutants are more resistant to stress than log-phase parental cells, suggesting that for *P. aeruginosa*, in contrast to *E. coli, rpoS* is not essential for stress survival (Jorgensen *et al.* 1999) and RpoS has more specific roles related to virulence and colonisation that must be integrated into the overall cell-density dependent expression of virulence determinants (Venturi 2003).

As it becomes clearer that quorum sensing is regulated not only by cell density but also growth phase, evidence suggests that the general stress response must play a key role in cell-to-cell signalling. It was initially reported that *rpoS* expression was not detected in certain mutants deficient in PAI-1 and PAI-2 production, indicating that both *lasR* and *rhl*R are required for full *rpoS* expression (Latifi *et al.* 1996). However, later studies revealed that in a *rpoS* null mutant, expression of *rhlI*, PAI-2 and r*hlR*-regulated virulence factors increased, whilst RhlR levels were unaffected. This suggested that RpoS was repressing *rhlI* activity and effectively ceasing production of PAI-2 and subsequently *rhlR*-associated virulence factors (Whiteley *et al.* 2000) and normal levels of RhlR indicated that this effect was not caused by direct inhibition of RhlR. The latter theory of *rpoS*-dependent regulation of QS is further consolidated by a study that has shown *rhlR*-controlled virulence factors elastase and pyocyanin are derepressed in a *rpoS* null mutant (Diggle *et al.* 2002).

The different functional roles played by RpoS as well as the involvement of other global regulators suggest that *Pseudomonas* spp. are predominantly regulated at a transcriptional level (Venturi 2003). For example, *rpoS* promoter activity is reduced by 90% in a null-mutant of the TetR-family regulator PrsA and the role of *rpoS* in the QS cascade is further complicated by PAI-1 and PAI-2 levels being unaffected by the reduced *rpoS* activity in the *prsA* mutant (Kojic and Venturi 2002). Furthermore, the two-component regulators GacA and GacS have been shown to regulate *rpoS*

transcription positively in *P. fluorescens* Pf-5 (Whistler *et al.* 1998) again supporting the influence of global regulators on the activity of *rpoS* in *Pseudomonas* spp.

1.2.3 RpoS and inorganic polyphosphate

Inorganic polyphosphate (poly P) is a linear polymer of hundreds of orthophosphate residues linked by ATP-like high-energy phosphoanhydride bonds. It is ubiquitous in nature, being detected in plants, animals, fungi and microbes (Kornberg *et al.* 1999) and performs a wide variety of roles including; a substitute for ATP, a chelator of divalent cations, a phosphate reservoir and an energy source (Chiang and Burrows 2003). Poly P is synthesised in bacteria by polyphosphate kinases (PPK) which catalyse the reversible transfer of the terminal phosphate residue of ATP to poly P (Ahn and Kornberg 1990). Poly P is a regulatory element in the stress response of *E. coli*, with PPK mutants demonstrating increased sensitivity to heat, oxidative and osmotic stress resulting from reduced *rpoS* expression (Rao and Kornberg 1996). Poly P also plays an important role in the virulence of *P. aeruginosa*, with PPK mutants demonstrating reduced flagella and pili-dependent motility (Rashid and Kornberg 2000), (Rashid *et al.* 2000a). Furthermore, PPK mutants are unable to form thick, differentiated biofilms and demonstrate reduced expression of virulence factors regulated by quorum sensing systems (Rashid *et al.* 2000b). As these factors are at least indirectly controlled by RpoS in *P. aeruginosa*, it could be suggested that, as in *E. coli*, the control of *P. aeruginosa* gene expression by poly P, is in part due to its regulation of *rpoS*.

Overall this research suggests that although *rpoS* is a master regulator of many genes involved in the stress response, it is itself controlled by a selection of global regulators that act in concert to allow *Pseudomonas* to thrive in such diverse environments. This ensures that the population has the flexibility to respond to the variety of stresses could be encountered from extremes of pH and desiccation to host attack and nutrient starvation

1.3 QUORUM SENSING in *P. aeruginosa*

The study of bacteria grown in planktonic culture has provided much valuable information regarding microbial physiology and molecular biology but in the natural, industrial and clinical environment it is becoming increasingly clear that bacteria predominantly grow in attached sessile communities termed biofilms (Costerton *et al.* 1999). Survival of an organism depends on its ability to monitor the environment and to respond accordingly and this becomes especially important in situations of starvation and attack. Microorganisms reside in environments with heterogeneous mixtures of species (the oral flora consists of up to 500 different bacterial species (Whittaker *et al.* 1996)) and so to compete effectively in a given environment, a population must be able to sense and respond to its own size and also that of its competitors.

Research into microbial cell-to-cell signaling was first investigated in the bacterium *Vibrio fischeri*, a symbiotic organism that displays a characteristic bioluminescence within its marine hosts. It was shown that the bacterium produced a diffusible molecule, which accumulated in the environment. At high cell densities a critical level was reached and the organism would luminesce (Nealson *et al.* 1978). This diffusible molecule was termed an "autoinducer" and was later characterised as *N*-3-(oxyhexanoyl)homoserine lactone (Eberhard *et al.* 1981). The cluster of genes which regulate luminescence was characterised as the *lux* operon and was cloned into *E. coli* (Engebrecht and Silverman 1984), revealing the molecular mechanisms of *lux* quorum sensing. At the top of the signaling cascade is a synthetase gene (*lux*I), which regulates the production of the small, freely diffusible autoinducers (AI). At high population densities the environmental accumulation of AI reaches a level at which the signal interacts with a transcriptional regulatory protein (luxR) which in turn activates the expression of a specific set of genes. (Parsek and Greenberg 1999). Therefore, a certain cell number or "quorum" is reached which allows the cells to determine and respond to the population density.

After *V. fiscerii*, the most characterised quorum sensing system is that of *P. aeruginosa* . It comprises two interacting networks termed the *las* and *rhl* systems, named after exoproducts they regulate (LasA/LasB and rhamnolipid respectively). The synthase gene *lasI* regulates production of the autoinducer *N*-(3-oxododecanoyl)-*L*-homoserine lactone (PAI-1) which interacts with the transcriptional regulator LasR; similarly *rhlI* regulates *N*-butyryl-*L*-homoserine lactone (PAI-2) production which interacts with the transcriptional regulator *rhlR*. Both *lasR* and *rhlR* regulate the production of a variety of virulence factors including LasB elastase, lipase, hydrogen cyanide, exotoxin A and pyocyanin. A high cell density is required to produce sufficient quantities of virulence factor to influence the surrounding environment (Parsek and Greenberg 2000) and so QS regulates both the secretion of exoproducts in response to a specific stress and ensures that the population density is sufficient to survive the host response to these virulence factors.

The *las* and *rhl* systems do not act independently; they represent a multilayed hierarchy with *lasR* exerting both transcriptional and posttranslational control on the expression of *rhlR* (Pesci and Iglewski 1998). Furthermore the RhlR-PAI-2 complex can control *rhlI* transcription (Latifi *et al.* 1996) and so this autoregulation of the *rhl* system provides yet another exquisite level of control. There are conflicting reports regarding the expression of *lasR* through the growth cycle; on the one hand it is reported to be expressed at a constant rate of synthesis throughout the cell cycle, including the stationary phase (Latifi *et al.* 1996). Conversely, reports indicate basal expression until the late log phase followed by maximal expression in stationary phase (Whiteley *et al.* 2000), the latter being more convincing due to the formers use of multicopy plasmids as opposed t chromosomal fusions which are more reflective of cellular levels. However, the expression of *rhlR* is less contentious and is reported to be expressed at a constant level until the onset of stationary phase, whereby promoter activity ceases (Alonso *et al.* 1999). Matters have been further complicated by the discovery of an additional AI compound, 2-heptyl-3-hydroxyl-4-quinolone or the *Pseudomonas* quinolone signal (PQS). Although PQS is regulated by *lasR* and *rhlR*, it can induce elastase production in a *lasR* mutant (Pesci *et al.* 1999) even though elastase production is directly under the control of *lasR*. Furthermore, the

quinolone signal shares strong structural homology with the 4-quinolone antibiotics, suggesting their efficacy is due to disruption of the quinolone signalling pathway.

A number of global regulatory genes have been revealed to play a dominant role in the QS hierarchy. The *P. aeruginosa Vfr* gene is a homologue of the *E. coli* cyclic AMP receptor protein (CRP), which regulates exotoxin A and CLP protease activity. The expression of *lasR* is significantly reduced in a *vfr* null (West *et al.* 1994), indicating that Vfr is above LasR in the QS hierarchy and is required for full LasR activity *in vitro*. The global regulator GacA is responsible for the production of antibiotics, toxins and exoenzymes in a range of *Pseudomonas* spp. In *P. aeruginosa* GacA activates the expression of *lasR* and *rhlR* and thereby modulates the production of PAI-2 and its exoproducts, i.e. pyocyanin, cyanide and lipase (Dacheux *et al.* 1994). Overexpresssion of the *P. aeruginosa* homologue of the *E.coli* dnaK mutation suppressor gene (*dnaK*) inhibits quorum-sensing dependent virulence factor production by repression of the *rhlI* gene (Branny *et al.* 2001) and is required for full translation of *lasb* and *rhlAB* (Jude *et al.* 2003). Random transposon mutagenesis of the *P. aeruginosa* genome revealed the *mvaT* gene is responsible for growth phase regulation of QS. In wild type PAO1 the QS-dependent gene *lecA* was not upregulated in late-exponential phase by the addition of either PAI-1 or PAI-2, however there was a significant increase in *lecA* expression at this growth stage when *mvaT* was inactivated (Diggle *et al.* 2002). This indicated that MvaT demonstrates a growth phase-dependent repression of LasR and RhlR activity, ensuring cells are approaching stationary phase before the QS cascade and consequent virulence factor production are upregulated.

QS is an adaptive mechanism which allows a community to both sense and respond to its own population and that of competitors. Research has shown that its regulation is not solely a product of high cell density but the coordination of numerous genetic pathways in response to environmental conditions. This further illustrates that bacteria can cooperate within communities ultimately for the benefit of the population.

1.4 BIOFILMS

1.4.1 Biofilm growth

Research into *rpoS* expression and quorum sensing in *P. aeruginosa* has been predominantly undertaken in planktonic culture using undefined medium such as LB broth. The ubiquitous nature of *P. aeruginosa* ensures growth in a diverse selection of environments which will not only be limiting for key nutrients but will present multiple stresses. Consequently, bacteria adapt by forming slow-growing, sessile communities termed biofilms, which constitute a major proportion of the bacterial biomass in nature (De Kievet *et al.* 2001). Bacterial biofilms are formed when unicellular organisms come together to form a community that is attached to a solid surface and encased in an exopolysaccharide matrix (Mah and O'Toole 2001).

The bacterial strain, the nature of the substratum and other environmental conditions all interact to determine the degree of adhesion of bacteria to a solid surface with strain properties being the dominant factor. However, the primary adhesion of bacteria to surfaces is governed by cell surface-surface and substrate-surface changes as well as hydrophobicities (Ahearn *et al.* 1999). A study by Sauer *et al.* (2002) subdivided the development of the *P. aeruginosa* biofilm into 5 distinct stages under continuous culture conditions. A) The primary event in formation of the biofilm is the reversible attachment of planktonic cells to the substratum with some cells remaining attached and others detaching after a short time. Once initially attached, a dynamic occurs with cells attaching, growing and detaching simultaneously and the equilibrium shifts towards rate of attachment in the early stages of biofilm formation (Lappin-Scott and Bass 2001). B) The second stage, termed irreversible attachment involves the formation of cell clusters by groups of cells in close proximity at the substratum. It is at this stage that the *las* QS system is also upregulated. C) The third stage of development, termed Maturation-1 is characterised by the progressive layering of the cell clusters and the upregulation of the *rhl* QS system. D) The Maturation-2 stage of biofilm development sees cell clusters reaching their maximum dimensions as cells begin to separate in preparation for dispersion. E) Finally cells from within the cluster demonstrate increased motility and move from within the

biofilm to disperse into the surrounding medium. Different modes of detachment have been noted with cells detaching from the biofilm as either single cells or thick clusters which differ in response to antimicrobials and colonisation of surfaces at different sites (Lappin-Scott and Bass 2001). Overall this suggests that bacteria demonstrate different phenotypes in relation to each stage of biofilm development and this is supported by results showing growth stage-dependent gene expression (Sauer *et al.* 2002).

1.4.2 Biofilms and infection

The prevalence of bacterial biofilms in the environment has ensured that they are a major cause of nosocomial infection, predominating in the hospital environment and in the tissues of infected patients (Costerton 2001) especially those involving burns, prosthetic surgery and cystic fibrosis (Gilbert and Brown 1995). Also, biofilms can form on medical implants such as catheters and contact lenses (Mah and O'Toole 2001). As cells grown in biofilms are reported to be 10-1000 times more resistant to antimicrobials than cells grown planktonically (Greenberg 1998) and the role of biofilm growth in nosocomial infection becomes more apparent, it is clear that efficient antimicrobial treatment is of the highest importance. The increased resistance of biofilms to a wide selection of antimicrobial agents cannot be attributed to solely to one mechanism, more a combination of factors that act in concert to increase the viability of the population. A distinguishing characteristic of the biofilm phenotype is the production of the exopolysaccharide matrix that coats the exterior of the community and acting as a barrier which isolates cells from fluctuations in the surrounding environment (Gilbert *et al.* 1990) and antimicrobial efficacy can be reduced by reaction with matrix components that limit the transport of agents to cells within the biofilm (Mah and O'Toole 2001). However, many antibiotics can diffuse readily through biofilms, albeit at a reduced rate of transfer (Stewart 1996). As the heterogeneity of the biofilm population ensures gradients of nutrients, waste products and oxygen, a cell's phenotype may depend on its individual position within the biofilm (Sternberg *et al.* 1999). Cells within the centre of the biofilm can be starved of oxygen and nutrients resulting in slow growth or dormancy (stationary phase growth) which has been shown to increase resistance to antibiotics

(Brown *et al.* 1988) and specifically, a combination of oxygen limitation and the presence of nitrate has been shown to increase biofilm resistance (Borriello *et al.* 2004). However, biofilm cells can also exhibit drastic changes in resistance to antibiotics through the exponential growth phase as well (Sloan *et al.* 2002). This suggests the heterogeneous nature of the biofilm structure involves various sub-populations of cells growing at different rates in relation to their position within the overall structure and this must be taken into account when investigating biofilms as a whole.

1.4.3 Biofilm global gene expression

Early transcriptional analysis of gene expression in biofilm growth was undertaken using insertional fusions or replicating plasmids (Wyckoff and Wozniak 2001). However, the recent availability of microarray technology with its ability to investigate bacterial responses at a genomic level has made these methods somewhat redundant. Surprisingly, *P. aeruginosa* microarray has revealed that as little as 1% of the genome demonstrated differential expression between planktonic and biofilm growth (Whiteley *et al.* 2001), whilst similar experiments in *E. coli* revealed a 2% difference in expression depending on growth phase. This suggests that the expression of a relatively limited numbers of genes is actually altered during biofilm growth.

1.4.4 Biofilms and RpoS

In *E. coli* RpoS demonstrates a repressive role on biofilm proliferation in log-phase growth (Corona-Izquierdo and Membrillo-Hernandez 2003) and in *P. aeruginosa* there is at least indirect evidence linking RpoS activity and the biofilm phenotype, primarily through the role of QS in biofilm development (Davies *et al.* 1998). Studies of the biofilm proteome using 2D PAGE have revealed that the *las* system is more influential in the later stages of development (Sauer *et al.* 2002) and regulates biofilm formation via regulation of the type IV pili expression (De Kievet *et al.* 2001). Furthermore, *lasR* mutant biofilms are more susceptible to kanamycin than wild-type biofilms which may be a result of increased antibiotic penetration due to a combination of reduced exopolysaccharide levels and thinner biofilm structures noted

in the mutant strains (Shih and Huang 2002). LasR has also been shown to regulate biofilm resistance to hydrogen peroxide by regulating catalase and superoxide dismutase expression (Hasset *et al.* 1999). In *P. aeruginosa rpoS* expression was found to be 3-fold higher in continuous-flow biofilms compared with stationary-phase planktonic culture, whilst immunoblot analysis revealed that the levels of RpoS protein were approximately the same (Xu *et al.* 2001). Studies using *P. aeruginosa* flagella mutants have suggested that motility is important for the initial stages of biofilm development by either providing sufficient force to ensure the cells are in contact with the solid interface or directly acting as an adhesin (O'Toole and Kolter 1998a). In the same study the use of type IV pili mutants indicated that the pilus regulates the differentiation of the initial bacterial monolayer into the complex microcolony. The involvement of type IV pili in biofilm growth is suggested to be mediated through twitching motility, which is required for normal biofilm formation. Mutants deficient in twitching motility form denser, more segregated colonies which indicates suggesting that type IV pili are also involved in the dispersion stages of biofilm development (Chiang and Burrows 2003).

Biofilm formation does not follow a single genetic pathway, moreover numerous inter-connected mechanisms exist which allow the organism to adapt to its environment. For example, the proteome of *P. aeruginosa* may be markedly different between static biofilms and flowing systems, in which the former is much more dependent on flagella motility in the colonisation stages of the biofilm development (Nealson *et al.* 1978). This is also demonstrated in non-motile and *clpP* mutant strains of *P. fluorescens* which are defective in biofilm formation when grown in a glucose minimal medium but form biofilms comparable to wild-type structures when grown using citrate or glutamate-based media (O'Toole and Kolter 1998b).

This consolidates that in any given environment the phenotype of the bacterium is greatly dependent upon nutrient availability either to alter gene expression so that alternative sources such as carbon can be utilised or the upregulation of acquisition systems in response to specific starvation. This is especially important when these factors are virulence determinants such as siderophores released in response to iron

limitation which can contribute to pathogenicity of *P. aeruginosa* in cystic fibrosis (Reid *et al.* 2002).

1.5 IMPORTANCE OF IRON FOR MICROBIAL GROWTH

1.5.1 Iron and the host-pathogen interaction

Although it is generally accepted that there are substantial levels of iron both humans and other animals, in reality iron is stored and maintained in ways that ensure almost no free iron is available to support bacterial growth, with only 10^{-18} M concentrations available in biological fluids (Bullen *et al.* 1978). In humans most iron is stored intracellularly in ferritin, haemosiderin or haem and extracellularly in body fluids it is attached to high-affinity binding proteins such as transferrin and lactoferrin (Bullen and Griffiths 1999). These proteins have a very high affinity for iron with association constants of around 10^{36} and furthermore these proteins are expressed at levels that ensure that they are only 30-40 % saturated (Bullen and Griffiths 1999). This provides a flexibility that can ensure a sudden influx of iron can be rapidly stored before being scavenged by invading bacteria. The production of a number of *P. aeruginosa* virulence factors is under complex regulation, including the level of available iron and *in vivo* environments such as the CF lung; iron availability is limiting (Heinrichs *et al.* 1991). Iron binding proteins such as transferrin and lactoferrin play a critical role in resistance to infection by limiting the availability of free iron and thereby slowing bacterial growth. Consequently to meet their iron requirement bacteria must compete with the host for an element which is tightly bound to these host proteins (Meyer *et al.* 1996). *P. aeruginosa* secretes a variety of extracellular virulence determinants in response to iron-limited conditions which include exotoxin A, elastase, alkaline protease, phospholipase C and leukocidin (Wick *et al.* 1990). Therefore the coordinated response of many bacteria to iron-limiting conditions not only involves the production of siderophores and the membrane receptors required for acquiring iron in the host environment but also the production of factors unrelated to iron metabolism such as bacterial toxins (Griffiths and Chart 1999).

1.5.2 Iron in solution

Iron is the second most abundant metal in the earth's crust and exists primarily in two oxidation states; ferrous (Fe^{2+}) and ferric (Fe^{3+}). In aqueous solution, at a neutral pH, ferrous iron is hydrated as a hexa-aqua iron Fe $(H_2O)_6^{2+}$ ion which has a solubility of 10^{-2} M, whilst ferric iron is hydrated as a Fe $(H_20)_6^{3+}$ ion with a solubility of 10^{-17}. The ease at which Fe^{2+} and Fe^{3+} undergo electron transfer and acid-base reactions explains the wide range of catalytic and biochemical functions of which the element is capable and underscores the importance of iron in biological systems (Crichton 1991). However, in aerobic conditions at neutral pH, molecular oxygen is reduced by ferrous iron in the Haber-Weiss reactions shown below producing toxic free radicals:

1) $Fe^{2+} + O_2 \leftrightarrows Fe^{3+} + O_2^{\bullet -}$
2) $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$
3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^-OH + {}^\bullet OH$

This is the oxygen paradox of aerobic organisms, in that they require molecular oxygen to survive yet free iron can generate free radicals toxic to the cell. This also means that in aerobic conditions iron is predominantly found in its insoluble ferric form, ensuring little free iron is available to organisms growing in an aerobic environment.

1.5.3 Iron autoxidation in growth media

The nature of iron in solution is highly dependent upon its ligand environment; any change such as pH, buffer or chelators will affect its reactivity. Autoxidation can be described as the oxidation of a substance by molecular oxygen in the absence of any cofactor and the mechanism of autoxidation of transitional metals such as Fe^{2+} is mainly dependent on reaction conditions (Tadolini 1987a). Therefore, care must be taken in choice of growth medium as the autoxidation of Fe^{2+} varies in the presence of different anions with marked differences between buffers regarding mechanism and rate of oxidation (Tadolini 1987b). This suggests that the availability of soluble and hence available iron may vary significantly between growth media and so what is assumed to be iron-replete conditions can in fact be limiting for iron. Ferrous iron

autoxidation also increases with the pH of the solution and even small changes in pH can dramatically affect iron solubility; however oxygen rich ligands such as phosphate can override the pH effect and promote oxidation at acidic pH (Welch *et al.* 2003).

1.5.4 Bacterial iron acquisition

The role of iron as an essential nutrient has ensured bacteria must employ a variety of mechanisms to acquire iron from the external environment. These systems include: a) enzymatic reduction of Fe^{3+} and subsequent transport of Fe^{2+} into the cell, b) the use of siderophores to chelate ferric iron and c) direct acquisition of iron from host sources such as transferrin and haem (Guerinot 1994). Therefore, for growth in an aerobic environment, organisms have developed specific systems for iron acquisition, sensing the level of iron ingested, terminating intake and sequestering toxic levels (Vasil and Ochsner 1999). Many microbial species acquire iron by the secretion of low-molecular mass, high-affinity chelators termed siderophores (Neilands 1973), whose synthesis is characteristically de-repressed only when microbial cells are limiting for iron. The siderophore is secreted into the external environment and forms a specific and highly stable complex with Fe^{3+} but displays a weak or negligible affinity for Fe^{2+} (Meyer and Abdallah 1978). In *P. aeruginosa* these acquisition mechanisms are the result of the expression of at least 200 genes regulated by iron-limitation (Ochsner *et al.* 2003) which are coordinated by the ferric uptake regulator (Fur). The *fur* protein acts in concert with iron as a co-repressor, in which the fur/iron complex binds to a "fur box" in the promoter sequence of iron-regulated genes, repressing their transcription. When iron concentrations become limiting, intracellular levels of Fe^{2+} drop below a certain threshold, de-repressing genes and consequently upregulating iron acquisition systems (Vasil and Ochsner 1999). Conversely, in iron-replete conditions *fur* can upregulate iron storage proteins such as bacterioferrins and oxidative stress defences such as catalase and peroxidase (Vasil and Ochsner 1999).

P. aeruginosa, due to its diverse environmental habitats, requires a range of mechanisms for acquiring and sequestering iron and principally expresses two siderophores, pyoverdine and pyochelin. There exists a hierarchy of expression which ensures that the chelator with the highest affinity for iron is preferentially expressed in the following order: pyoverdine>pyochelin. Pyoverdine is secreted into the external environment in response to iron starvation, exclusively chelating Fe^{3+} and the resulting complex is termed a ferrisiderophore. The iron chelate then binds to the ferripyoverdine-specific outer-membrane receptor, FpvA (Visca *et al.* 2003) and is transported across the outer membrane into the periplasm where it is translocated into the cytoplasm through a TonB1 dependent mechanism (Bullen *et al.* 1974).

At neutral pH, pyoverdine can be detected spectrophotometrically at 402 nm and fluorometrically at 398 nm excitation and 470 nm emission (Meyer and Abdallah 1978) and there is an inverse relationship between the iron concentration of the medium and the amount of pigment synthesised after entry into stationary phase. This characteristic absorbance is not displayed when the pigment is complexed with iron and so levels of bound ferric iron can be calculated from the absorption of the cell-free culture supernatant at 402 nm. *P. aeruginosa* can also use low affinity aquisition systems involving functional siderophores such as pyruvate, malate, citrate and isocitrate. In *Pseudomonas* spp. citrate can act as an iron shuttle transporting Fe^{3+} to the cell envelope, whereby the complex dissociates and iron enters the cell via an unknown mechanism (Harding and Royt 1990). Citrate can also be used as a carbon source by *Pseudomonas* spp. and at high concentrations inhibits pyoverdine synthesis (Harding and Royt 1990). This further consolidates the hypothesis of a chelator hierarchy, with *P. aeruginosa* using the acquisition system with the highest affinity for iron in any given conditions. A ratio of 20:1 between citrate and iron is required for efficient chelation indicating that it is a low-affinity acquisition system (Spiro *et al.* 1967) and so very high concentrations of citrate are required for it to surpass the chelating ability of pyoverdine.

1.5.5 Iron-dependent virulence factors

As well as acquisition systems, *P. aeruginosa* secretes a series of virulence factors in response to iron-limited conditions. Exotoxin A (ETA) is directly under the control of the Fur regulatory system and consequently iron-limitation is a major stimulus for ETA synthesis (Bjorn *et al.* 1978). Innate defence mechanisms of the host normally impose bacteriostatic conditions by maintaining essential nutrients at concentrations insufficient for bacterial growth (Cox 1980). Therefore, to obtain sufficient iron bacteria must compete with the host for an element which is tightly bound to host proteins, which include ferritin, transferrin, lactoferrin and haemoglobin (Stintzi *et al.* 1996). Also when unsaturated, these iron-binding proteins appear to play a significant role in resistance by slowing down bacterial growth (Bullen *et al.* 1974). In iron-limited environments such as macrophages and cell vacuoles, bacteria release factors to disrupt the host defence and in these conditions siderophores such as pyoverdine and pyochelin play an important role in *P. aeruginosa* pathogenicity especially in disorders such as cystic fibrosis where the lungs of CF patients are likely to be iron limiting due to the presence of high amounts of mucus containing lactoferrin (Wang *et al.* 1996). Exotoxin A and elastase are also important determinants of virulence in the chronic lung infection model and *P. aeruginosa* actively synthesises them in a low iron medium, indicating that the iron concentration of the culture medium can influence the pathological progression of the *P. aeruginosa* infection (Sokol and Woods 1984). The alternative sigma factor, PvdS, regulates pyoverdine production and is shown to be transcribed in the CF lung (Hunt *et al.* 2002) and is also responsible for the regulation of ETA production in *P. aeruginosa*, via *regAB* and *toxA*, (Ochsner *et al.* 1996). Pyochelin increases the lethality of virulent bacteria solely by the promotion of bacterial growth (Cox *et al.* 1981) and *P. aeruginosa* can also utilise the siderophores of other bacteria such as enterobactin of *E. coli* (Liu and Shokrani 1978).

Such a variety of iron-dependent virulence determinants illustrates that a single starvation such as iron can be responsible for the upregulation of genes that are not even directly involved with its acquisition but are involved in adaptation to a host environment in which the starvation predominates and so accurate quantification of

expression of these genes would reveal how such mechanisms interact and ultimately discern to what extent they responsible for the pathogenicity of *P. aeruginosa* in a variety of disorders from wound infections to cystic fibrosis.

1.6 QUANTIFICATION OF GENE EXPRESSION

Bacterial genes are expressed at varying levels during the cell cycle and regulated by many factors including growth, differentiation and stress. Therefore accurate quantification of gene expression can reveal how an organism responds to its environment. This becomes especially important for the investigation of human pathogens, as monitoring the expression of key virulence determinants can reveal vital information regarding both the process of the infection and can identify potential candidates for antimicrobial treatment. A wide selection of gene expression techniques are now available which allows the selection of methods best suited to the requirements. Broadly, techniques fall into three categories: amplification techniques, hybridisation techniques and gene fusion technology.

1.7 NUCLEIC ACID HYBRIDISATION TECHNIQUES

1.7.1 Northern blotting

Northern blotting was originally adapted from the Southern method of DNA blotting (Southern 1975) and was the first technique to accurately quantify RNA. RNA samples are denatured using agarose gel electrophoresis and then transferred to nitrocellulose membranes. The immobilised RNA is then hybridised to a radiolabelled probe which is then quantified by blot detection (Thomas 1980). Northern blotting remains an accurate method to determine mRNA size and integrity; but it does lack sensitivity, with a minimum of $1x10^5$ to $1x10^7$ copies of target required for accurate detection (Sambrook *et al.* 1989).

1.7.2 S1 nuclease assay

The S1 nuclease assay is used to determine intron and exon sites, locate of 5' and 3' termini and to quantify mRNA levels. In experimental conditions conducive to DNA:RNA hybrid formation, double stranded DNA is denatured in the presence of mRNA forming a duplex between the DNA exons and the mRNA and any non-duplex DNA is hydrolysed by the addition S1 nuclease. The remaining duplex is then denatured and the fragment size is visualised using Southern blotting, permitting the

identification of intron:exon boundaries and mRNA termini (Sharp *et al.* 1980). Although more sensitive than Northern blotting, the S1 nuclease method still requires a minimum of $5x10^5$ to $5x10^6$ copies of target sequence for accurate detection.

1.7.3 The RNase protection assay

The RNase protection assay employs a RNA probe system to map and quantify mRNA. The probe is generated *in vitro* and hence circumvents the need for isolation of rare RNA species. A plasmid-cloning vector is constructed containing a viral SP6 promoter sequence immediately upstream from a polylinker. Target sequences are then cloned into the vector, which will be exclusively transcribed by SP6 RNA polymerase, forming a radiolabelled anti-sense probe for the target sequence. This probe anneals to target sequences forming a RNA:RNA duplex, which is subsequently digested with RNase and visualised by denaturing polyacrylamide gel electrophoresis (Melton *et al.* 1984). This method can be used to quantify mRNA and to map sequence termini and intron boundaries and is 25 times more sensitive than the S1 nuclease method, requiring approximately $2x10^4$ copies of target sequence (Melton *et al.* 1984).

1.7.4 *in situ* hybridisation

One of the most sensitive hybridisation techniques is *in situ* hybridisation, whereby cells are lysed *in situ* on nitrocellulose filters to which the resulting DNA becomes non-covalently attached. After the application of radiolabelled probes, the location of the mRNA within the cell can be identified. Although this method does possess a limit of detection of 10 to 100 copies of target per cell, it is technically difficult and does not lend itself to large-scale sample processing (Ferre *et al.* 1994).

There are fundamental problems associated with hybridisation methods when applying them to bacterial gene expression. Firstly, bacterial mRNA is inherently unstable and many genes are expressed at very low copy numbers and so would not be quantified or even detected using these methods. Secondly, these methods are very time consuming and may not be applicable necessarily to screening large numbers of samples. Therefore other techniques are required which provide sensitive

levels of detection but also whereby gene expression can be quantified using rapid and simple methods which minimise stress to the cell.

1.8 GENE FUSION TECHNIQUES

A reporter gene can be described as a gene fusion encoding a readily measurable phenotype that can be distinguished easily over the background of endogenous proteins (Alam and Cook 1990). By attaching response elements to reporter genes, alterations in reporter gene activity can provide a way of selectively monitoring gene expression (Naylor 1999).

1.8.1 *lac* fusions

Undoubtedly, the role of the *lac* operon as the foundation of molecular biology is beyond reproach and its simplicity and versatility has ensured its incorporation into the genomes of practically every genetically tractable organism (Alam and Cook 1990). Beyond their role in selective screening, *lacZ* fusion constructs provided the blueprint for all subsequent reporter technologies and are constructed in two distinct ways. Transcription or operon fusions comprise a *lac* operon downstream of a promoter but do not include the gene's specific ribosomal binding site (RBS). This ensures that the signal generated from the *lac* operon only reflects the transcription of the gene of interest and any post-transcriptional effects only represent expression of wild type *lacZ* (Hand and Silhavy 2000). Translational or protein fusions comprise both the RBS and the promoter of the gene of interest fused to a functional fragment of *lacZ*, which ensures the fusion sequence is in-frame. As the protein itself is a functional hybrid any post-transcriptional regulation of the gene of interest will be reflected in the activity of β-galactosidase (Hand and Silhavy 2000). By using a combination of these fusion proteins, the transcription, translation and degradation of any chosen gene can be measured. Unfortunately, the colorimetric versions of the β-galactosidase assay display poor sensitivity and a narrow dynamic range (Naylor 1999) and are by nature an invasive technology requiring cell lysis prior to assay which prevents visualisation of reporter activity within the cell. However, recent advances have taken the principals of fusion technology provided by *lacZ* and applied them to reporters which are less invasive and display higher sensitivity.

1.8.2 Luciferase fusions

Luciferase enzymes are characterised by their ability to catalyse the oxidation of certain substrates such as luciferin and coelenterazine, resulting in light emission (Naylor 1999). Luciferase fusions can be separated into two main types depending on experimental requirements. Firefly luciferase was originally cloned from *Photinus pyralis* and transformed into *E. coli* (De Chial *et al.* 1985). To emit light the enzyme requires the addition of the substrate and ATP, as illustrated below:

$$_{D}\text{-luciferin} + ATP + O_2 \leftrightarrows \text{oxyluciferin} + AMP + \text{pyrophosphate} + \text{light}$$

Bacterial luciferase was first characterised as the *luxCDABE* operon originally isolated from the bioluminescent marine bacteria *Vibrio harveyi* and *Vibrio fischeri* (Engebrecht *et al.* 1983), (Belas *et al.* 1984), then later from the soil bacterium *Photorhabdus luminescens* (Winson *et al.* 1998). The *luxAB* genes code for the luciferase enzyme, whilst *luxCDE* codes for the substrate (a long-chain fatty acid). Light is produced via the oxidation of a riboflavin phosphate cofactor (Roda *et al.* 2003) illustrated below:

$$2)\ FMNH_2 + O_2 + RCHO \leftrightarrows FMN + RCOOH + H_20 + \text{light}$$

Bacterial luciferase fusions have a distinct advantage in that the substrate is produced intracellularly. Ironically, firefly luciferase requires the addition of exogenous luciferin but its sensitivity is far higher than its bacterial counterpart (Naylor 1999). However, both types of luciferase fusions demonstrate greater sensitivity than *lac* fusions and their non-invasive monitoring allows the visualisation of gene expression at a cellular level.

1.8.3 Green fluorescent protein

The green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* has become one of the most widely studied and exploited proteins in biochemistry and cell biology (Tsien 1998). The purified protein consists of 238 amino acid residues, of which the Ser-Tyr-Gly residues at position 65-67 of the peptide form the *p*-hydroxybenzylideneimidazoleinone chromophore. The absorbance spectrum of the wild type protein is characterised by a major excitation peak at 395 nm and an emission peak at 508 nm (Heim *et al.* 1995). By fusing *gfp* to a known promoter sequence, the expression of a gene can be monitored by detection of green fluorescence either spectrofluorometrically or visualised by epifluorescence microscopy and laser scanning confocal microscopy (Valdivia *et al.* 1996). The success of GFP-based systems relies on the fact that the protein requires no additional gene products from *Aequorea victoria* besides molecular oxygen and the chromophore formation is not species specific (Heim *et al.* 1995). However, the wild-type protein is very stable and so cannot be used to monitor transient gene expression. This problem is circumvented by the addition of a short C-terminal oligopeptide extension, which renders the protein susceptible to degradation by endogenous intracellular proteases and significantly reduces the half-life of the protein compared with the wild type (Andersen *et al.* 1998). Another problem associated with the wild type protein is its low intrinsic fluorescence. This is primarily due to inefficient folding at temperatures above 27°C, leading to large amounts of insoluble protein. However, certain mutations in the fluorophore increase the folding efficiency of the protein and consequently the sensitivity of detection (Scholz *et al.* 2000). For example, the *gfp*mut3 mutant of green fluorescent protein contains a double substitution of S65G and S72A and when expressed in *E. coli* fluoresces with approximately 100-fold higher intensity than wild-type GFP and the time taken for the fluorophore to mature is also significantly reduced (Chalfie *et al.* 1994). Although gene fusion technology has made possible both the visualisation and quantification of gene expression at levels of sensitivity far higher than hybridisation techniques it still remains difficult to monitor very low expression levels using these techniques and it become clear that to accurately quantify very low concentrations of mRNA, target sequences require amplification.

1.9 AMPLIFICATION TECHNIQUES

1.9.1 Polymerase Chain Reaction (PCR)

The influence of PCR on the progress of molecular biology cannot be understated and remains the technique of choice for the measurement of gene expression. Its exquisite sensitivity and relative simplicity has ensured that it can easily detect mRNA at levels previously unobtainable using other methods This was initially achieved by using the reverse-transcriptase polymerase chain reaction (RT-PCR). Once RNA is isolated from a cell, the sequence of interest can be reverse transcribed to produce a cDNA copy of the gene. Two specific primers are designed which anneal to the cDNA target in the presence of a DNA polymerase and dNTPs. The duplexes are then denatured and act as primer for the next cycle of annealing and denaturing and so the amount of product doubles with each subsequent cycle (Sharp *et al.* 1980). This technique has distinct advantages compared to other hybridisation and gene fusion techniques. Firstly, it demonstrates very high sensitivity, easily detecting 10-100 copies of target from a cell in a mixed population and theoretically detection of 1 copy is possible making possible the quantification of previously undetectable, rare and low copy number mRNAs. Secondly, it is possible to use this technique for a large number of samples and the technique is less variable and time consuming than its hybridisation counterparts. Real-time PCR combines both rapid PCR cycling and continuous monitoring of product formation (Turner *et al.* 1995). A fluorescent dye is included in the reaction mixture which only binds to double stranded DNA (i.e. cDNA generated from target mRNA in the reverse transcription step). This means that the fluorescence generated by each reaction is directly proportional to the amount of product. Usually, a threshold fluorescence value is pre-determined and high target concentrations require fewer cycles of amplification to reach the threshold than low concentrations (Bustin 2000) and so generation of fluorescence in conjunction with suitable RNA controls allows quantification of product at each cycle of amplification. This presents a more accurate representation of RNA levels than the end-point detection of standard RT-PCR (Edwards and Saunders 2001). PCR is an amplification technique and so is susceptible to errors produced by amplification of contaminating DNA present in target samples. Furthermore, small tube-to-tube

differences at the initial stages of the assay can grossly affect the final yield of PCR products (Wilson 1997) but even considering these problems PCR-based techniques remain the most sensitive methods for quantifying gene expression.

1.9.2Signal-Mediated Amplification Reaction Technology (SMART)

The SMART technique was developed by Cytocell Ltd as a novel isothermal nucleic acid amplification assay which is both a rapid and sensitive method for RNA and DNA detection (Wharam *et al.* 2001). The assay is based upon a set of oligonucleotide probes termed the extension probe and the template probe, which are designed for a specific 60 base-pair sequence of the target nucleic acid. In optimised conditions the two probes anneal both to the target sequence and each other, forming a three-way junction (3WJ), (Fig 1.2 A.). At this stage the two probes provide a binding site for DNA polymerase which transcribes the template probe sequence forming a double stranded functional promoter site for T7 RNA polymerase. This in turn generates a target-dependent RNA signal (RNA-1), (Fig 1.2 B.). In the second stage of the assay, RNA-1 anneals to an amplification probe, again forming a promoter site for T7 RNA polymerase, which generates a second, amplified RNA signal (RNA-2) (Fig. 1.3).

Fig. 1.2 Formation of 3WJ and subsequent amplification of signal. **A.** Extension and template probes anneal to the target sequence, forming a functional DNA polymerase promoter. The template probe contains a non-functional single-stranded (ss) RNA polymerase promoter sequence. DNA polymerase extends the extension probe to form a functional double stranded (ds) RNA polymerase promoter. **B.** RNA polymerase binds to the promoter and transcribes multiple copies of primary RNA species (RNA-1) from the template probe.

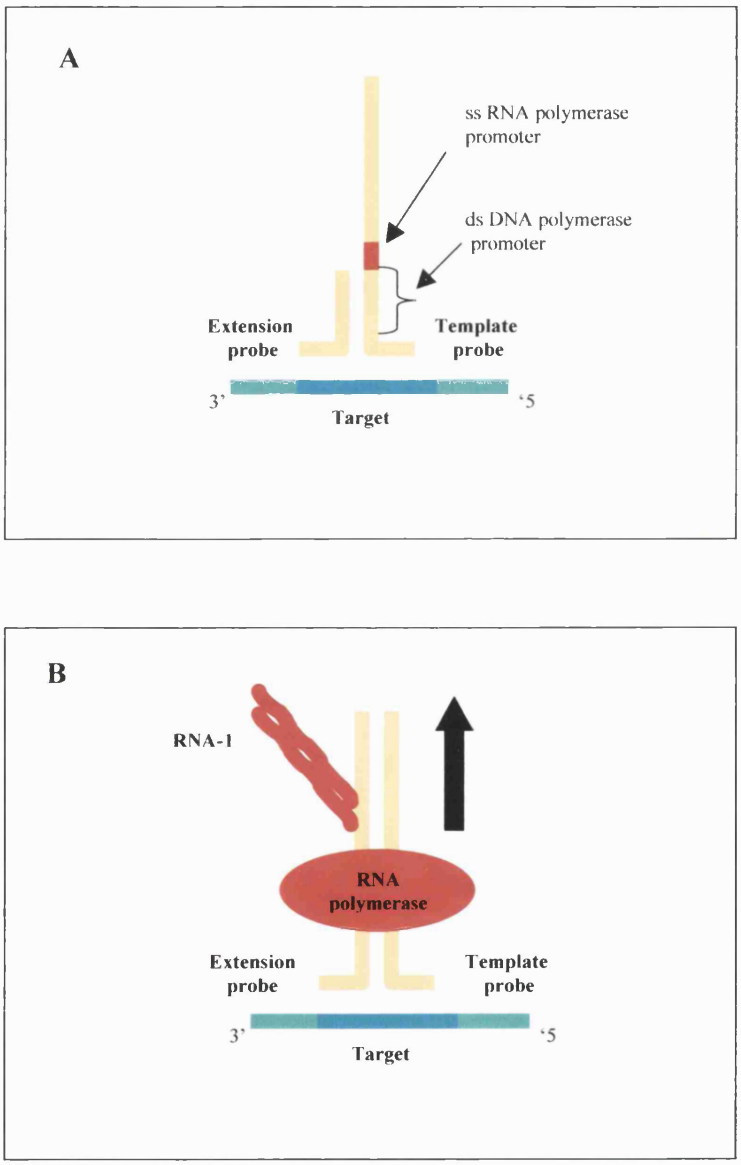
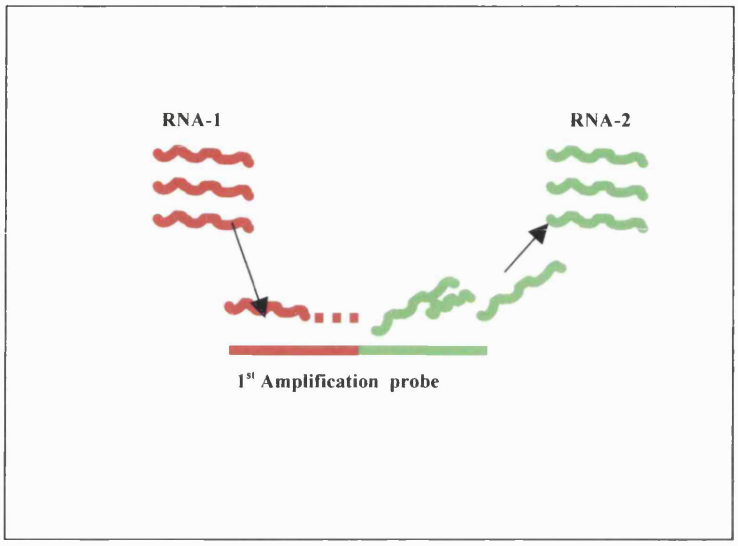
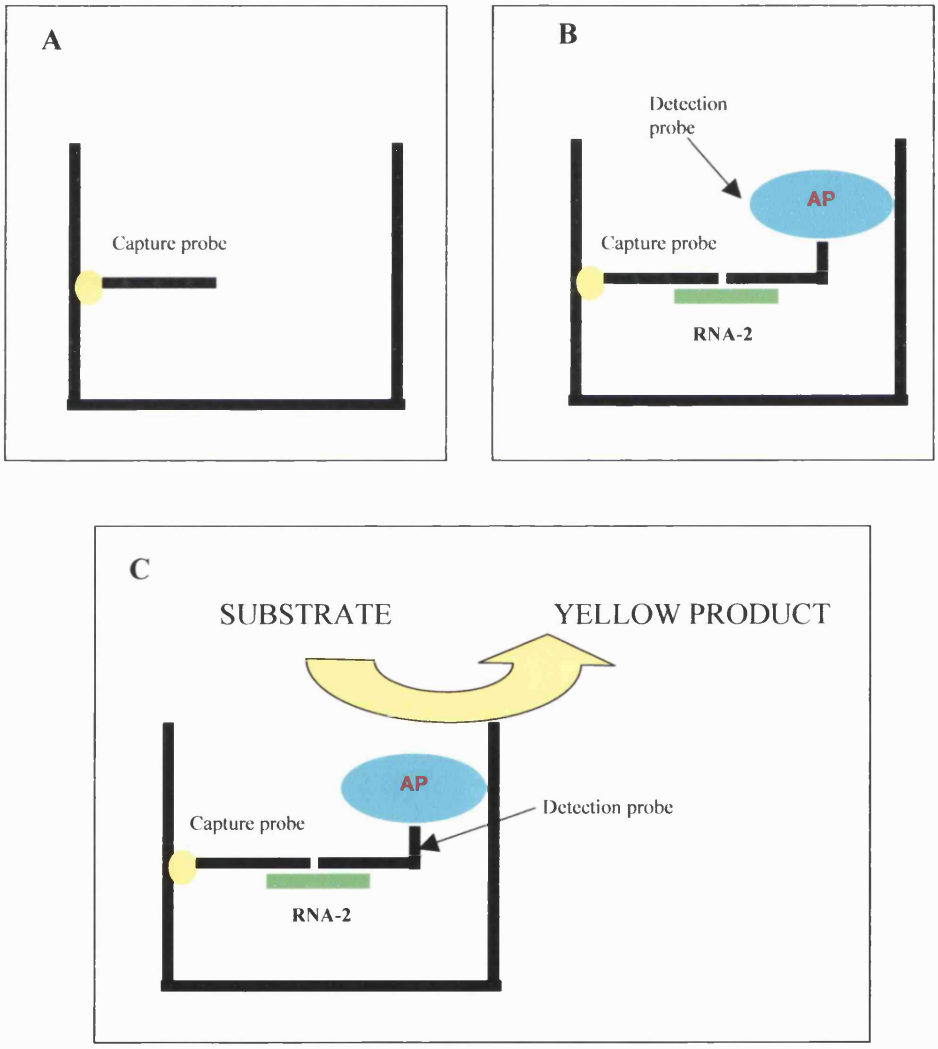


Fig 1.3 SMART assay amplification step. RNA-1 anneals to the amplification probe and is extended and transcribed by DNA and RNA polymerases to form an amplified secondary RNA species (RNA-2).



This signal is then quantified using the enzyme-linked oligosorbent assay (ELOSA). The ELOSA end detection method initially involves the binding of a capture probe to the streptavidin coating of a 96-well plate (Fig. 1.4 A.). Then, RNA-2 anneals both to the capture probe and an alkaline phosphatase-labeled detection probe (Fig. 1.4 B.). A washing step is employed to remove any unbound probe and then the substrate is added which is converted to a yellow product by alkaline phosphatase (Fig 1.4 C.). The change in colour is monitored at 405 nm using a spectrophotometer. This technique allows multiple samples to be quantified at the same time and the specificity of this technique ensures that any signal generated by the assay is due solely to the presence of RNA-2 (Wharam *et al.* 2001).

Fig. 1.4 RNA quantification by Enzyme-Linked Oligosorbent Assay (ELOSA). **A.** A biotinylated capture probe, complementary to RNA-2 binds to the streptavidin-coated microtitre plate. **B.** RNA-2 produced by the amplification step, anneals both to a capture probe and to an alkaline phosphatase (AP) associated detection probe. **C.** Wash steps remove any unbound probe then 4-nitrophenylphosphate is added as a substrate for AP. A yellow colour is produced and absorbance is measured at 405 nm. A standard curve is created using known RNA-2 concentrations which are quantified in each assay. This used to calculate the amount RNA-2 produced in each target reaction.



The SMART assay is able to detect target signal in as little as 100 pg of *E. coli* K12 total RNA (Wharam *et al.* 2001) and 250 ng of marine cyanophage genomic DNA (Hall *et al.* 2002) and although it is not as sensitive as RT-PCR the SMART assay does have distinct advantages. Firstly, the 3WJ complex will form only in the presence of the target sequence and so contaminating nucleic acid will not be amplified in the reaction, ensuring the assay is truly target-dependent. Consequently, SMART can be used to detect target sequences in crude cell lysates (Hall *et al.* 2002), (Wharam *et al.* 2001). Secondly, the SMART assay is undertaken at one temperature and so expensive thermocycling equipment is not required (the assay can be even conducted in a hot block) making it more conducive to high-throughput applications. Finally, the SMART assay is based on the amplification of a signal not the nucleic acid target which means that the assay can easily be adapted to quantify different nucleic acid targets (Wharam *et al.* 2001).

The SMART assay has been used predominantly as a detection technique and its potential as a quantitative method has not been explored. Its minimum sensitivity in *E. coli* has been demonstrated and the assay would have to be optimised to the required sensitivity of other amplification techniques. However, if successful, SMART could become a simple, reproducible and target-specific method of measuring gene expression.

1.10 AIMS OF THE STUDY

The aims of the study were to:

- Optimise the SMART assay for measurement of gene expression in *P. aeruginosa* using plasmid-borne and chromosomal *rpoS*::*gfp*mut3 fusions.

- Confirm an iron-replete phenotype is achievable when cells are grown in CDM_{10} complete and if this is not possible this medium will be modified to ensure growth can be truly defined.

- Assess direct fluorescence measurement as a technique to monitor gene expression in *P. aeruginosa* plasmid-borne and chromosomal *gfp*mut3 fusion strains and then to use this method to determine the effects of glucose, magnesium and iron limitation on the expression of *rpoS* in planktonic and biofilm culture.

2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS, PLASMIDS AND GROWTH CONDITIONS

The microorganisms used in this study are listed in Table. 2.1. All strains were maintained at -80°C in 20 µl aliquots of Luria-Bertani (LB) broth containing 30% glycerol. Fortnightly, strains were streaked onto LB agar containing the appropriate antibiotic, incubated at 37°C for 16 h and then stored at 4°C until required. Bacteria were routinely grown in 20 ml of LB, at 37°C in baffled 250 ml conical flasks, and were shaken at 200 rpm in a Controlled Environment Incubator Shaker (New Brunswick) unless otherwise stated. The following antibiotic concentrations were used in this study: ampicillin, 100 µg/ml for *E. coli*; carbenicillin, 300 µg/ml for *P. aeruginosa*.

Table. 2.1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
P. aeruginosa		
PAO1	Prototroph	Suh *et al.* 1999
SS336	*rpoS*109::*gfp*mut3; translational fusion in the chromosome	S.J. Suh
SS429	PAO1/pSS127	S.J. Suh
SS431	PAO1/pSS129	S.J. Suh
AT101	PAO1/pIAPX20	This study
AT102	PAO1/pIA101	This study
E. coli JM109		Lab collection
Plasmids		
pSS127	*rpoS*109::*gfp*mut3; translational fusion in pUCP19; Ap^r/Cb^r	S.J. Suh
pSS129	*rpoS*109::*gfp*mut3; transcriptional fusion in pUCP19; Ap^r/Cb^r	S.J. Suh,
pUCP20	Broad host range vector derived from pUC18/19; Ap^r/Cb^r	West *et al*, 1994
pUCP20-*gfp*mut3	pUCP20 containing *gfp*mut3 gene	Dacheux *et al.* 2001
pIA101	pUCP20-*gfp*mut3 with deletion of the *plac* –10 region	Dacheux *et al.* 2001
pIApX20	pIA101-derived plasmid containing the *gfp*mut3 gene under a constitutive pX20 promoter	D. Dacheaux

Ap^r: ampicillin resistance, Cb^r: carbencillin resistance

Note:

The *rpoS*109 fragment consists of the *nlpD* gene and the first 22 amino acids of the *rpoS* gene (Suh *et al.* 1999)

All *gfp* fusions contain the *gfp*mut3 gene (Cormack *et al.* 1996)

2.1.1 Preparation of Chemically Defined Media (CDM)

The components of CDM_{10} complete are listed in Table 2.2. Chemicals were obtained for SigmaAldrich, St. Louis, USA unless otherwise stated. All nutrients are added at excess concentrations so that a planktonic population can be grown to a maximum theoretical optical density of 10. Therefore, in nutrient-limited conditions the entry of cells into stationary phase would be solely in response to starvation of the specific nutrient. Stock solutions were made at 10 x concentrations in double-distilled water (ddH_2O). The pH of the MOPSO solution was adjusted to 7.8 with 10 M NaOH and then filter sterilised using sterile disposable 500 ml filter units (Nalgene). All other solutions were sterilised by autoclaving for least 15 min at 121°C. Components were mixed in appropriate proportions for each experiment. In later experiments, MOPSO buffer was replaced by 0.1 M phosphate buffer (Sambrooke and Russell, 2001) and consequently no additional phosphate source was required.

Table 2.2 Components of the *P. aeruginosa* chemically defined medium CDM_{10} complete

Component	Concentration (mM)
Glucose	40.00
KCl	0.62
NaCl	0.50
$(NH)_4.SO_4$	0.50
$MgSO_4.7H_2O$	40.00
MOPSO*	50.00
$KH_2PO_4.3H_2O$	3.20
Fe $SO_4.7H_2O$†	0.01
ddH_2O to 1l	

* MOPSO = 3-(N-Morpholino)-2-hydroxy-propanesulfonic acid

† Acidified with 1 ml H_2SO_4 per 1000 ml to prevent precipitation.

2.1.2 Inoculation and growth of planktonic cultures

The inoculation procedure for planktonic culture was as follows; a single colony from an LB-agar stock was added to 20 ml of LB in a 250 ml conical flask and incubated at 37°C for at least 6 h. A starter culture was prepared by diluting these cultures 1:500 in 20 ml of pre-warmed CDM followed by incubation for 16 h at 37°C. After measuring OD at 470 nm, cultures were diluted to a low cell density in 20 ml of pre-warmed CDM and then incubated at 37°C until required. When investigating nutrient limitation, starter cultures contained limiting concentrations of the appropriate nutrient to ensure no carry over into the test culture. Calibration curves for nutrient limitation experiments were constructed by plotting final stationary-phase cell density against nutrient concentration. A concentration range of 0 to 100 mM was used for glucose and 0 to 1000 μM for magnesium chloride. The linear portion of the plot revealed concentrations at which cell density is limited by the nutrient concentration, above this range cell density was limited by other factors.

2.1.3 Inoculation and growth of biofilm cultures

Biofilm cultures were grown by the nutrient-depleted biofilm method (Buhler *et al.* 1998). Briefly, purified agar (Oxoid) was dissolved in sterile ddH_2O water to a 1.2% w/v concentration and then autoclaved at 121°C for at least 15 min. The other components of the CDM were mixed in a sterile 500 ml Duran bottle. A 12.5 ml volume of both the CDM and purified agar were added to a sterile, 30 ml polystyrene universal tube and mixed by gentle inversion. A 20 ml volume of this mixture was added to a sterile petri dish and left to set. Plates were then over-dried for 1 h at 37°C. A sterile cellulose nitrate membrane with a hydrophobic ring (45 mm diameter, 0.2 μM pore, Sartorius) was placed on a vacuum filter unit and was washed through with 5 ml of CDM (minus limiting nutrient where appropriate) and then placed at the centre of the agar plate.

Starter cultures were incubated for 16 h at 37°C in 20 ml of CDM. Cells were re-suspended in fresh CDM to an OD of 0.02 and 50 μl of this culture were pipetted onto the centre of a pre-warmed membrane which corresponded to 1 x 10^6 cells per membrane. Membranes were then incubated at 37°C until required. At the required time point membranes were removed from the agar and placed in a 30 ml polystyrene universal tube containing 5 ml of CDM and were then vortexed for 30 seconds. The OD of the suspensions was measured at 470 nm and cell number per membrane was calculated. Aliquots of biofilm suspension were examined microscopically for aggregates using a Nikon Optiphot-2 light microscope at x 60 magnification.

Calibration curves for nutrient limitation studies were determined by growing biofilms on CDM agar containing various concentrations of the specific nutrient at 37°C for 48 h and then calculating cell density as described previously. Final cell density was plotted against nutrient concentration. Concentration ranges used were from 0 to 100 mM and 10 to 1000 μM for glucose and magnesium respectively. The linear portion of the plot reveals concentrations at which cell density is limited by the nutrient concentration, above this range the cell density is limited by other factors.

2.1.4 Measurement of fluorescence of *gfp*mut3 fusion strains through the growth cycle

To measure the expression of *rpoS* under nutrient limitation the fluorescence of various *rpoS*::*gfp*mut3 fusions were measured using *P. aeruginosa* PAO1 as a control for intrinsic fluorescence. Starter cultures were grown in the appropriate medium as described in section 2.3 and cells were then re-suspended to a low density in pre-warmed medium. At hourly intervals, 3 ml aliquots of the culture were removed and added to a 4 ml disposable cuvette. Fluorescence was measured at 480 nm excitation and 511 nm emission and OD measurements were made at 470 nm. The fluorescence of growth medium was subtracted from sample values. The fluorescence of *P. aeruginosa* PAO1 was plotted against OD at 470 nm and the equation of the line was calculated. This allowed the fluorescence of PAO1 and any *gfp* fusion to be directly compared at a specific cell number.

2.2 RNA ISOLATION AND QUANTIFICATION

2.2.1 Isolation of total RNA from *P. aeruginosa* PAO1

Cell RNA was isolated using the RNeasy mini-Kit (Qiagen Ltd, Crawley, West Sussex) as described by the manufacturer's protocol. Briefly, bacteria were harvested by centrifugation at 5000 *g* for 5 min at 4°C. The supernatant was removed and the pellet was re-suspended in TE buffer containing 400 μg/ml lysozyme. Buffer was added each sample, followed by centrifugation for 2 min at 12,000 *g*. A volume of 100% ethanol was added to the supernatant and the sample was applied to a mini-column and centrifuged at 8000 *g* for 15 sec. DNA was removed from the sample by incubation at room temperature with 30 Kunitz units of DNase I. The column was washed by another buffer/centrifuge step and then total RNA was eluted into RNase-free water and then stored at -20°C until required.

2.2.2 Quantification of total RNA

RNA samples were quantified by a fluorescence-based assay using Ribogreen reagent (Jones *et al*, 1998). Briefly, Ribogreen reagent (Molecular Probes Europe Ltd., Leiden, Netherlands) was diluted to a concentration of 2μg/ml in TE buffer and added to a sterile, 4 ml disposable cuvette. A standard curve was constructed using *E. coli* total RNA, diluted to 1000, 500, 100, 20 and 0 μg/ml in TE buffer. Assay and standard curve samples were added to the Ribogreen reagent and incubated at room temperature for 5 min, protected from light. Fluorescence values were measured at 480 nm excitation and 520 nm emission. The fluorescence of the reagent blank was subtracted from sample values and RNA concentrations were determined from the standard curve.

2.3 TRANSFORMATION OF PLASMIDS INTO *P. aeruginosa* PAO1

The minimum sensitivity of the SMART assay of *gfp*mut3 in cellular RNA had not been determined and so initial research was undertaken using *P. aeruginosa* PAO1 containing high copy number plasmids, which would provide large amounts of target RNA for assay optimisation. Three plasmids were selected containing *gfp*mut3 sequences fused to, a) a constitutive promoter (pIAPX20), b) a scrambled promoter (pIA101*) and c) an inducible p*lac* promoter (pUCP20), (Dacheux *et al.* 1994). Plasmids were first transformed into *E. coli* by heat shock, then extracted and transformed into *P. aerugi*nosa by electroporation.

2.3.1 Transformation of *E. coli* JM109 by heat shock

Vials of competent *E. coli* JM109 cells were removed from storage at -80°C and thawed at room temperature. A 100 μl volume of cell suspension was added to 5 μl of plasmid sample and a negative control. Tubes were then chilled on ice for 20 min. A water bath was pre-heated to 43°C in which cells were then heat shocked for 45 sec then immediately placed onto ice for 2 min. Each sample was inoculated into a 30 ml polystyrene universal tube containing 1 ml of LB broth. Tubes were then incubated at 37°C for 1 h. A 100 μl sample of this culture was spread onto an LB-agar plate containing 100 μg/ml ampillicillin and then incubated at 37°C for 16 h.

2.3.2 Isolation of plasmid DNA

E. coli JM109 cultures containing the plasmid were incubated at 37°C for 16 h in LB broth containing 100 μg/ml ampicillin. Plasmid DNA was isolated from these cultures using Promega Wizard mini-prep extraction kit in accordance with manufacturer's protocol. Samples were then stored at -20°C until required.

2.3.3 Transformation of *P. aeruginosa* by electroporation

Electroporation was carried using a sucrose gradient centrifugation method (Smith and Iglewski 1989). Briefly, *P. aeruginosa* PAO1 cultures were incubated for 16 h at 37°C in LB broth. Cells were re-suspended in pre-warmed medium to and OD of 0.1 and then grown to early log phase OD of 0.5. Cells were harvested by centrifugation

at 10,000 *g* for 10 min at 4°C. The pellet was re-suspended in an equal volume of 300 mM sucrose solution, centrifuged again and then re-suspended in a 0.5 volume of sucrose. A final centrifugation step was undertaken and the cells were finally re-suspended in a 0.01 volume of 300 mM sucrose. The suspension was chilled on ice for 30 min prior to electroporation. Plasmid samples were re-suspended in molecular-grade water to a concentration of 1 μg/ml. A 40 μl volume of *P. aeruginosa* cells was mixed with 5 μl of plasmid solution then transferred to chilled 0.2 cm gap cuvettes. Exponential decay pulses were generated by a Biorad Laboratories gene pulsar at the following parameters: 1.60 kV at a capacitance of 25 μfm. This equated to a time constant of between 4.8 and 5 mS. Samples were re-suspended in pre-warmed LB broth and incubated at 37°C for 16 h. Successful transformation of *P. aeruginosa* cells with *gfp*mut3 plasmids was confirmed by fluorescence microscopy. Culture aliquots were then stored at -80°C in 25% LB/glycerol.

2.4 ISOLATION OF OUTER MEMBRANE PROTEINS

Starter cultures of *P. aeruginosa* PAO1 were incubated at 37°C for 16 h in 250 ml conical flasks using 20 ml of the appropriate medium. Cultures were then inoculated into 20 ml of pre-warmed medium and incubated at 37°C for 3 h. Cultures were then inoculated into 200 ml of pre-warmed medium and incubated at 37°C for 5 h in 2000-ml conical flasks. The resultant cultures were then transferred to 500 ml polypropylene centrifuge tubes and spun at 8000 *g* at 4°C for 10 min using a Beckman ultracentrifuge. Supernatants were removed and the pellet was re-suspended in 20 ml of sterile 0.9% saline and then spun at 8000 *g* at 4°C for 10 min in a 50 ml conical centrifuge tube (Greiner). Supernatants were removed and cells were re-suspended in 6 ml of saline. Cells were lysed using a Constant Cell Disruption Systems cell disrupter. The resulting lysates were spun at 6000 *g* at 4°C for 5 min and supernatants were decanted into fresh tubes. *N*-lauryl sarcosine was added to a final concentration of 2% and tubes were incubated at room temperature for 1 h. Lysates were then transferred to 200 ml polycarbonate ultracentrifuge tubes and spun at 20000 *g* for 1 h at 4°C. The supernatants were removed and the outer membranes were then resuspended in 100 µl of sterile water and stored at -20°C until required. Protein concentration was calculated using the Lowry assay (Lowry *et al.* 1958) using BSA as a reference for the standard curve.

2.5 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was undertaken as previously described for a 10% gel (Sambrooke and Russell, 2001) using a Mini Protean III gel kit. The resolving gel was poured and a layer of water was added to flatten the surface. After 20 min the water was poured away and remaining liquid was blotted using filter paper. The stacking gel was poured onto the resolving gel, the comb was added and the gel was left for 20 min to set. Protein samples were mixed with 3x loading buffer (150 mM Tris-Cl, 300 mM dithiothreitol, 6% SDS, 0.3% bromophenol blue, 10% glycerol) to a final concentration of 10 μg/μl and then heated at 100°C for 8 min. Samples were loaded onto the gel in 5 μl volumes in conjunction with 10 μl of MW standard (Invitrogen) and run at 200V/cm for approximately 30 min in Tris-EDTA buffer (Sigma). The gel was removed from the apparatus and stained with Brilliant Blue R concentrate for 30 min. Excess stain was poured off and the gel was transferred to a de-stain solution (50% v/v methanol, 40% v/v H_2O, 10% v/v acetic acid) for a minimum of 6 h. Gels were then dried using an Invitrogen gel drying kit.

2.6 MEASUREMENT OF PYOVERDINE PRODUCTION

Pyoverdine levels of planktonic culture were calculated as described by Wilderman *et al* (Wilderman *et al.* 2001) which is a modified version of that originally described by Meyer and Abdallah (Meyer and Abdallah 1978). Briefly, bacteria were grown to an OD of 0.5 at 405 nm in the appropriate medium and then centrifuged for 5 min at 8000 *g*. The resulting supernatants were normalised for differences in cell density and absorbance was measured spectrophotometrically at 402 nm. Pyoverdine concentration was calculated using the following formula: $M=\Delta/\varepsilon$.

Where, M = Molar concentration

Δ = Absorbance at 405 nm

ε = Extinction coefficient of pyoverdine (1.9×10^{-4} $M^{-1}cm^{-1}$).

2.7 SMART ASSAY

2.7.1 Bioinformatic junction design

SMART assay probes were designed as follows: gene target sequences for 23S rRNA and *gfpmut3* were obtained from Genebank (http://www.ncbi.nlm.nih.gov/Genbank), pasted into a Microsoft Word document and converted into Fasta format. A 4 base pair (bp) sequence within the target gene was selected to be the site of the 3WJ (three-way junction). A 120 bp synthetic target probe was designed containing the 58 bp sequences flanking the site of the 3WJ and extension and template probes were designed containing a 30 base-pair sequence (termed the foot), complimentary to the target RNA sequence.

Stem loops in the secondary structure of target mRNA can greatly reduce the efficiency of 3WJ formation, therefore sequences which possess high ΔG values are favoured as they reduce steric hindrance and increase the specificity of probe annealing. Probes were assessed for stem loops using mfold (http://.bioinfo.math.rpi.edu/~mfold/rna/form1.cgi) under the following parameters: 150 mM NaCl, 6 mM $MgCl_2$ and annealing temperature of 41°C (representing the SMART assay conditions). The annealing profiles of the extension and template probes were checked using GeneRunner software; as sequences which avoided base-pair interactions can increase the efficiency of the junction formation. Template probes were also checked for secondary structure stem loops using the mfold program. Once the probes were designed, the annealing temperatures of both template and extension probes were calculated to ensure compatibility (http://www.nwfsc.noaa.gov/protocols/oligonucleotidestmcalc.html). A high cellular concentration, in conjunction with constant expression during the cell cycle indicated that 23S rRNA could be used as an accurate internal standard for the expression of target genes. Therefore SMART probes were designed for 23S rRNA using the methods described above. Probes were also designed for the gene encoding the green fluorescent protein mutant, *gfpmut3*. This would be used to quantify the expression of a variety of *gfp* fusion constructs, allowing the investigation of a large variety of genes without requiring the design and construction of probes for each new target.

2.7.2 SMART assay three-way junction (3WJ) step

This step involves the formation of a 3WJ in the presence of target and the subsequent generation of RNA signal (Fig. 1.1 A). All SMART assays were performed using master mixes in order to minimise variation between samples. Oligonucleotide probes were obtained from Oswel DNA service (University of Southampton, UK) and probe sequences are listed in Appendices 1.1 and 1.2. A master mix was made up, per reaction, as follows: 10 fmol extension probe; 5 fmol template probe; 50 mM NaCl; 100 ng of *M. lysodeikticus* genomic DNA; 2 μl of 10x transcription buffer (Ambion Europe Ltd., Huntingdon, Cambridgeshire) and RNase-free water was added to a final volume of 15.3 μl. The mixture was aliquotted into 0.2 ml thin-walled thermocycler tubes containing either 5 fmol target probe (test reaction) or *M. lysodeikticus* genomic DNA (control reaction). The tubes were heated in a Perkin Elmer GeneAmp PCR System 2400 thermocycler at 95°C for 5 min (denaturation step) followed by 41°C for 30 min (annealing step). An enzyme mix containing, per reaction: 8.5 mM rNTPs and 20 μM dNTPs (Amersham Pharmacia biotech Inc., Piscataway, NJ); 240 units T7 RNA polymerase, (Ambion Europe Ltd) and 4 units *Bst* DNA polymerase (New England Biolabs, Beverly, MA) was added to each tube. Samples were heated at 41°C for a further 2 h (annealing and transcription steps).

2.7.3 SMART assay amplification step

This involves the amplification of the signal produced in 3WJ step (Fig. 1.1 B). The assay was undertaken as described for the 3WJ step but using the following probe concentrations: 5 fmol extension probe; 1 fmol template probe, 50 amol target probe. On completion of the assay temperature was reduced to 37°C and an amplification mix was added containing, (per reaction): 20 fmol amplification probe; 400 μM rNTPs; 20 μM dNTPs; 4.5 μl of 10x transcription buffer; 154 units T7 RNA polymerase; 4 units *Bst* DNA polymerase and RNase-free water was added to a final volume of 25 μl. Tubes were then heated at 37°C for a further 2 h

2.7.4 Template probe assay

This assay determines how efficiently the primary RNA species (RNA-1) is transcribed from the template probe (Fig. 3.5). The complement probe anneals to the template probe to form a functional T7 RNA polymerase promoter in the absence of a target sequence, from which the primary RNA signal is transcribed, amplified then quantified. A reaction mix was made up as follows: 1 fmol complement probe; 400 μM rNTPs; 2 μl transcription buffer; 154 units T7 RNA polymerase and RNase-free water was added to a final volume of 15 μl. The mix was then aliquotted into 0.2 ml thin-walled thermocycler tubes containing either 5 fmol template probe (test reaction) or *M. lysodeikticus* genomic DNA (control reaction) and then tubes were incubated at 37°C for 3 h.

2.7.5 SMART assay "one-pot" method

This modified version of the standard assay involves the combination of the transcription and annealing steps into one addition, effectively reducing the overall assay time to from 4 h to 2.5 h. The initial master mix was made up as specified in the amplification step protocol. The enzyme mix was made up, per tube, as follows: 5 mM rNTPs; 5.5 μM dNTPs; 2 μl transcription buffer; 394 units T7 RNA polymerase; 8 units *Bst* DNA polymerase and RNase-free water to a final volume of 20 μl. Tubes were placed in a thermocycler and heated at 95°C for 5 min (denaturation step) followed by 41°C for 1 h (annealing and transcription step). The amplification probe and enzyme mix were added to each tube. Tubes were then incubated at 41°C for a further 90 min (amplification step). On completion of the assay, samples were kept at -20°C until required.

2.7.6 Enzyme Linked Oligosorbent Assay (ELOSA)

ELOSA is the detection method used for all versions of the SMART assay (see Fig. 1.3) in which a standard curve of known RNA signal concentrations is used to quantify the RNA signals generated in the assay. A hybridisation mix was made up as follows (per well): 3 pmol detection probe; 0.9 pmol capture probe and 140 μl of hybridisation buffer (0.5M EDTA, 1M NaCl, 50 mM Tris base, 0.1% w/v BSA, pH to 8.0). The hybridisation mix was added to each well of a streptavidin coated 96-well

plate (Thermo Life Sciences, Hampshire, UK). Standard curve probe was added at 200, 140, 70, 35, 17 fmol with RNase-free water used as a blank; 10 μl of assay sample were added to the reaction tubes. The plate was sealed with an adhesive film to prevent contamination and shaken for 30 min at 300 rpm. After shaking, the plate seal was removed and the well contents were discarded. Unbound probe was removed by washing the wells three times with 200 μl of wash buffer (1x TBS, 0.1% Tween 20). The substrate, 4-nitrophenylphosphate, was made up at 5 mg/ml in alkaline phosphatase buffer (Boehringer Ingelheim GmbH Germany) and 200 μl was added to each well. Colour change at 405 nm was recorded at 2 min intervals for 32 min at 37°C using a Dynatech MR5000 plate reader with Biolink software. Results for the standard curve probes were plotted as A_{405}/min against probe concentration and were used to quantify RNA transcript in the assay samples.

2.7.7 Additional amplification assay

The additional amplification assay comprises a SMART assay with a secondary amplification step (see Fig 3.11). The assay was conducted as specified for the SMART one-pot assay with the addition of 20 pmol of secondary amplification probe to the enzyme mix. The signal levels of the samples were quantified by ELOSA using probes designed to detect the product of the secondary amplification step.

2.7.8 Facilitator assay

Facilitator probes are designed to anneal to the target, adjacently to the template and extension probes. This increases the stability of the 3WJ, reduces non-specific annealing and therefore increases assay sensitivity. The protocol for the one-pot assay was followed, adding 200 and 500 fmol of facilitator probes to the master mix. Assay sensitivity using both individual and tandem administration of facilitators was investigated.

2.8 STATISTICAL ANALYSIS

All experiments were conducted on at least 3 separate occasions and the significance difference between test and control values was calculated using an unpaired t-test assuming equal variance and a one-tailed distribution. Results were considered significant if $P \leq 0.05$.

3 OPTIMISATION OF THE SMART ASSAY TO MEASURE GENE EXPRESSION IN *P. aeruginosa* PAO1

3.1 INTRODUCTION

The SMART technique was developed by Cytocell Ltd to be a rapid and sensitive method for DNA detection. The assay was originally designed for the detection of DNA targets for use in medical diagnostics but it has also been shown to detect 16S rRNA reproducibly in total RNA extracts of *E. coli* K12 (Cormack *et al.* 1996). However, its potential for quantifying gene expression has not been fully investigated.

The basic theory of the technique is outlined in Fig 1.2. Briefly, two oligonucleotide probes termed the extension probe and the template probe are designed for a specific 60 base-pair sequence within the target gene. In the presence of the target sequence the two probes come together to form a 3-way junction (3WJ). At this stage the two probes form a binding site for DNA polymerase. The enzyme binds to the complex and copies the template probe sequence, forming a double stranded binding site for T7 RNA polymerase. This enzyme then transcribes the primary RNA species (RNA-1) from the extension probe sequence (See Fig 1.2). In the second stage of the assay, RNA-1 anneals to an amplification probe and again forms a T7 RNA polymerase binding site from which the secondary RNA species (RNA-2) is transcribed (see Fig 1.3). This signal is then quantified by the enzyme-linked oligosorbent assay (ELOSA (Fig. 1.4). Assay conditions are optimised to ensure that the template and extension probes only come together in the presence of a target sequence. This ensures that the production of RNA signal and its subsequent amplification and detection only can occur in the presence of a specific target. This effectively eliminates errors produced by amplification of contaminating DNA, which can be a common problem in PCR experiments (Wilson 1997).

The green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* has become one of the most widely studied and exploited proteins in biochemistry and cell biology (Tsien 1998). The success of GFP-based systems relies on the fact that the protein requires no additional gene products from *Aequorea victoria* and the chromophore formation is not species specific (Heim *et al.* 1995). Therefore, by fusing *gfp* to a promoter sequence, the expression of a gene can be monitored by detection of green fluorescence either spectrofluorometrically, or visualised by epifluorescence microscopy (Valdivia *et al.* 1996). The *gfp*mut3 mutant of green fluorescent protein contains a double substitution of S65G-S72A and when expressed in *E. coli*, the protein fluoresces with a 100-fold higher intensity than wild type GFP (Andersen *et al.* 1998). By designing probes for the detection of *gfp*mut3, the SMART assay could be used to quantify the expression of a variety of *gfp* fusions constructs. This would allow the investigation of the expression of a large variety of genes without requiring the design and construction of SMART probes for each individual target. Furthermore, gene expression could be also confirmed by direct fluorescence measurements during technique development stages of the study.

Sample-to-sample variation in the quantification of mRNA is a common occurrence in both RT-PCR and the SMART assay, especially when samples are taken from different cultures; errors can be compounded and levels of gene expression can be misinterpreted. This can be addressed by amplifying, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values could be normalised (Karge *et al.* 1998). A high cellular concentration, in conjunction with a reported constant expression during the cell cycle (Bustin 2000) suggested that 23S rRNA would be a useful positive control for the SMART assay.

Hopefully the SMART assay could be optimised to improve its overall sensitivity and be further developed for the quantification of mRNA targets as currently it has only been used to detect rRNA and DNA targets in *E. coli* (Wharam *et al.* 2001). By adapting the assay to quantify mRNA SMART could be applied to investigate gene expression in *P. aeruginosa*. In particular the effect of nutrient starvation on the expression of *rpoS* as this has been indicated to play an important role in the overall

pathogenicity of the organism. It would be naive to assume that the SMART assay could reach the exquisite sensitivity of LightCycler PCR but a level of sensitivity that would permit the quantification of plasmid-borne and chromosomal gene fusions could be achievable. This coupled with target specificity and simple methodology could prove SMART to be a functional and accurate assay of gene expression.

The aims of this study were to:

- To establish if the SMART assay could be used to detect gene expression reproducibly in *P. aeruginosa*. Initially, the assay would be optimised to detect 23S rRNA for use as a positive control. Once established, the SMART assay would be optimised for the detection of *gfpmut3* mRNA, allowing investigation of gene expression of numerous *gfp* fusions.

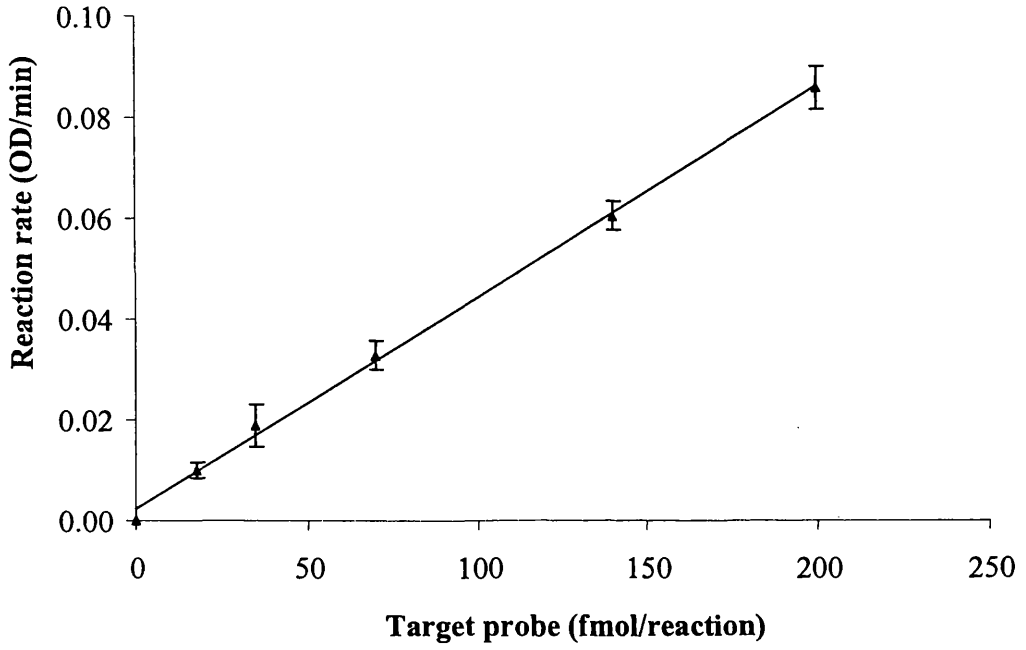
- To determine the sensitivity of the SMART assay using plasmid and chromosomal *rpoS*::*gfp*mut3 fusion strains and the capacity of the SMART assay for future gene expression studies investigating nutrient starvation and *rpoS* would be explored.

3.2 GENERAL SMART ASSAY OPTIMISATION

3.2.1 ELOSA standard curve

The Enzyme Linked Oligosorbent Assay (ELOSA) is a probe-based detection method using a 96-well format, which quantifies the RNA species produced by the SMART assay (see Fig 1.3). The ELOSA involves the association of a biotinylated capture probe with an alkaline phosphatase-associated detection probe, both of which contain sequences complementary to the SMART assay product, RNA-2. The capture probe binds to the streptavidin coating of the 96 well plate and then RNA-2 anneals the capture probe and the detection probe together. Unbound probe is removed by several of washing steps. The substrate, 4-nitrophenylphosphate is added to each well and the action of alkaline phosphatase produces a colour change at 405 nm which is detected spectrophotometrically. Sample levels were calculated using a standard curve of known RNA-2 concentrations run in each ELOSA. Studies undertaken by Cytocell have confirmed that in order to calculate sample concentrations accurately, 200 fmol of standard curve probe should produce an OD/min value between 0.060 and 1.0. Fig. 3.1 is a representative standard calibration curve and illustrates that a rate of approximately 0.9 OD/min is produced by 200 fmol of standard curve probe and a linear relationship between reaction rate and RNA transcript exists at a target probe range of 25-200 fmol.

Fig. 3.1 Calibration curve for the ELOSA assay, measuring reaction rate as a function of target probe concentration. A standard curve was produced by measuring absorbance at 405 nm of reactions containing a range of standard curve probe concentrations. n=3, ± SD.



3.2.2 Template probe assay

The 3WJ step of the SMART assay involves template and extension probes annealing in the presence of a target sequence to form a 3WJ. DNA polymerase uses the extension probe sequence as a primer to synthesise a complementary strand to the template probe. This forms a double stranded complex, from which RNA-1 is transcribed. The efficiency of the template probe transcription can be assayed by replacing the extension probe with a complement probe. The complement probe is an extension probe which lacks the "foot" sequence, so it can anneal to the template probe in the absence of a target sequence (see Fig 3.2). Consequently, RNA transcript production directly correlates with the efficiency of template probe transcription as opposed to the amount of target sequence in the sample.

Fig 3.2 Assay of template probe transcription. A. The complement probe anneals to the template probe forming a double stranded RNA polymerase promoter. **B.** RNA polymerase transcribes the template probe sequence, producing RNA-1 which can be then amplified and quantified using the standard SMART assay protocol. Therefore, RNA transcript produced correlates with how efficiently the template probe is transcribed by RNA polymerase.

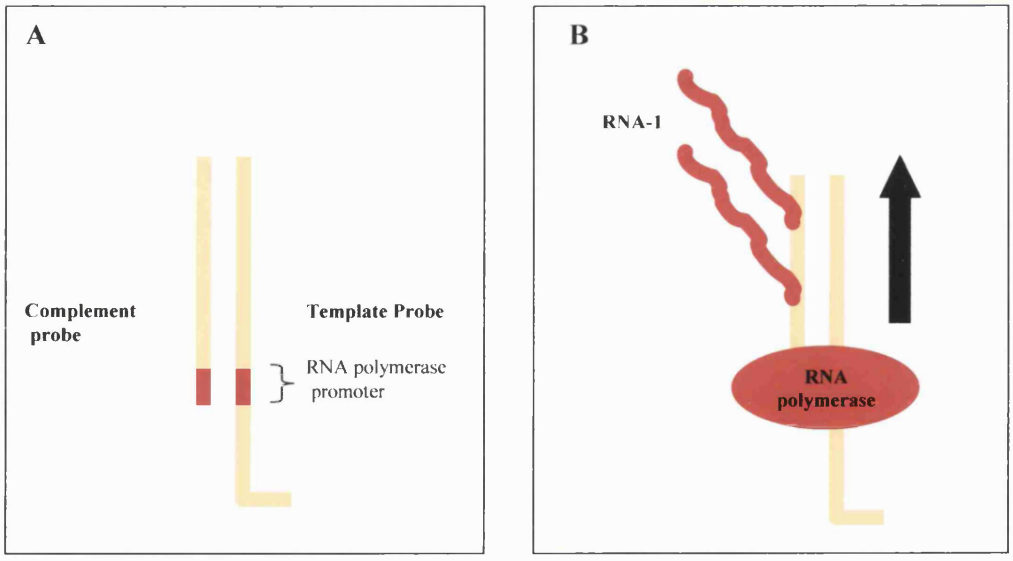
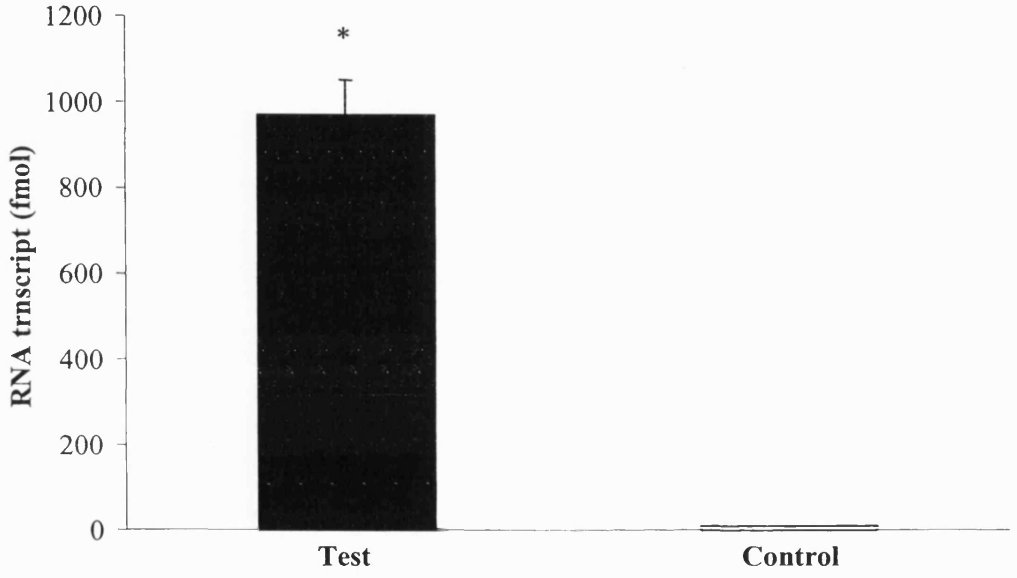


Fig. 3.3 illustrates that 5 fmol of template probe produced 971 fmol of RNA transcript, whilst the negative control produced 8 fmol RNA transcript, corresponding to a very a high signal-to-noise ratio of 125:1. This confirmed that the template probe was being transcribed efficiently; producing high levels of RNA transcript with minimal background amplification and indicated that signal generated by the assay was not the result of non-specific transcription from the template probe, which would be reflected by high levels of amplification in the control assay.

Fig 3.3 Determination of the efficiency of template probe transcription. The efficiency of template probe transcription was determined by quantifying the transcript produced using a complement probe which anneals to the template probe, generating a RNA signal in the absence of a target sequence. Black bars indicate the amount of RNA transcript detected using 5 fmol of template probe. White bars indicate background amplification generated by the assay in the absence of complement probe. n=3 ± SD.



* P ≤0.05

3.3 OPTIMISATION OF SMART ASSAY FOR DETECTION OF 23S rRNA

3.3.1 The SMART assay 3WJ step

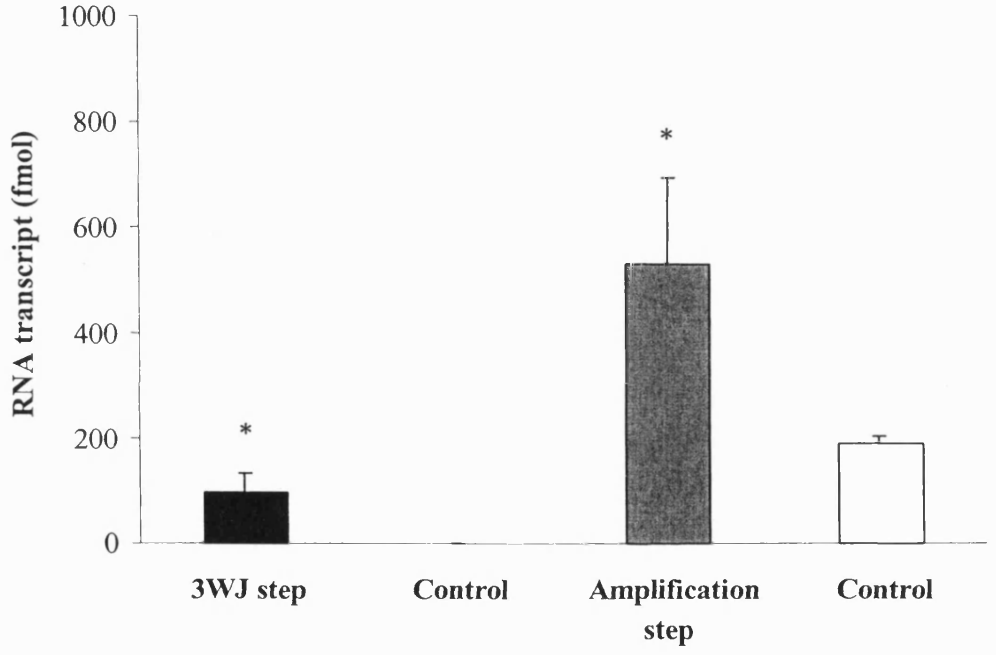
An assay of the 3WJ step of SMART is predominantly used in probe optimisation experiments to quantify production of RNA-1. RNA-1 is produced by the 3WJ in the presence of a specific target (3WJ step) and then amplified by a linear probe forming RNA-2 (Amplification step) which is then quantified by the ELOSA step. The amount of RNA transcript produced by the 3WJ step is detectable, but the level of amplification is not sufficient for gene expression experiments. However, this step does provide a point of comparison between newly-designed probes and if problems are encountered within the SMART assay then it can help identify in which step the problem is occurring.

Fig 3.4 illustrates that 5 fmol of synthetic 23S rRNA target produced 98 fmol of RNA transcript, indicating an approximate 20-fold amplification of signal. No background amplification was detected in the negative control reactions. This indicated that the 3WJ step of the SMART assay was working efficiently using the probes designed for a 23S rRNA target; however the amplification step is required to provide an increase in assay sensitivity.

3.3.2 The SMART assay amplification step

The next stage in SMART assay optimisation was to determine the efficiency of the amplification step. RNA-1 produced by the 3WJ only step is further amplified by a linear probe forming a secondary RNA species (RNA-2); this is called the amplification step. Fig. 3.4 indicates when the SMART assay was conducted using the amplification step, 50 amol of synthetic 23S rRNA target produced 527 fmol of RNA transcript, whilst control reactions produced 189 fmol. This represented an approximate 10,000-fold amplification of the target. The signal-to-noise ratio of 3:1 was higher than the minimum acceptable value of 2:1, indicating that the amplification step greatly increases the sensitivity of the SMART assay.

Fig. 3.4 Quantification of the RNA species produced by the SMART assay. The primary RNA species of the SMART was produced by the 3WJ step using 5 fmol of synthetic *P. aeruginosa* 23S rRNA target (black bar). The secondary RNA species of the SMART assay was produced by the amplification step, using 50 amol of synthetic *P. aeruginosa* PAO1 23S rRNA target (grey bar). Background amplification for both assays was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bars). n=3 ± SD.



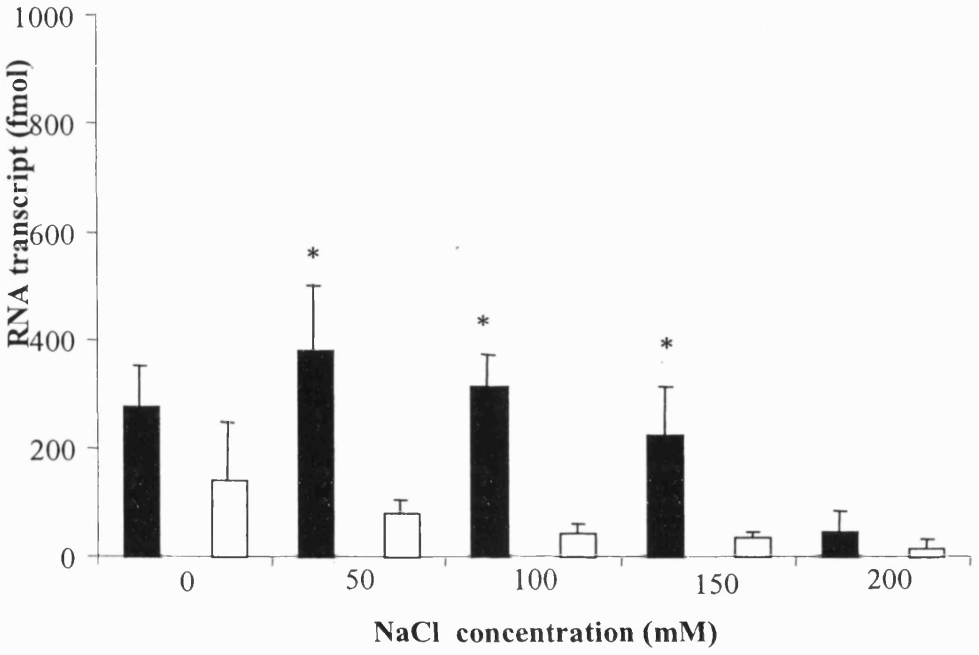
* P ≤ 0.05

3.3.3 Optimisation of salt concentration for SMART assay of 23S rRNA target

Salt concentration is a key factor in nucleic acid hybridisation reactions. In initial experiments a 50 mM concentration of NaCl was used, however optimal salt concentration for the SMART assay varies between target sequences. Therefore the SMART assay of a synthetic *P. aeruginosa* 23S rRNA target was undertaken at NaCl concentrations of 0, 50, 100, 150 and 200 mM. The ideal salt concentration would be determined by a high level of amplification in target reactions and low levels of amplification in control reactions. This corresponds to a high signal-to-noise ratio.

Fig. 3.5 shows that target amplification levels were reduced by increasing NaCl concentration. The SMART assay using 0 mM NaCl produced the worst signal-to-noise ratio of 2:1, whilst at 50 mM and 100 mM, ratios were 5:1 and 7:1, respectively. A 150 mM concentration produced lower levels of RNA transcript, but a high signal-to-noise ratio of 6:1 and 200 mM concentration of NaCl produced a low level of transcript and a signal-to-noise ratio of 3:1. A 100 mM salt concentration was selected for further studies as it produced the highest signal-to-noise ratio of 7:1 and a high level of amplification (6500-fold). Although a 50 mM salt concentration provided the higher level of amplification (7500-fold) it was not sufficient to justify using a concentration which produced a lower signal-to-noise ratio (5:1).

Fig. 3.5 SMART assay of a synthetic *P. aeruginosa* 23S rRNA target, conducted at a range of salt concentrations. SMART assay was undertaken at a range of salt concentrations, using 50 amol of synthetic *P. aeruginosa* 23S rRNA synthetic target (black bars). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bars). n=3 ± SD.

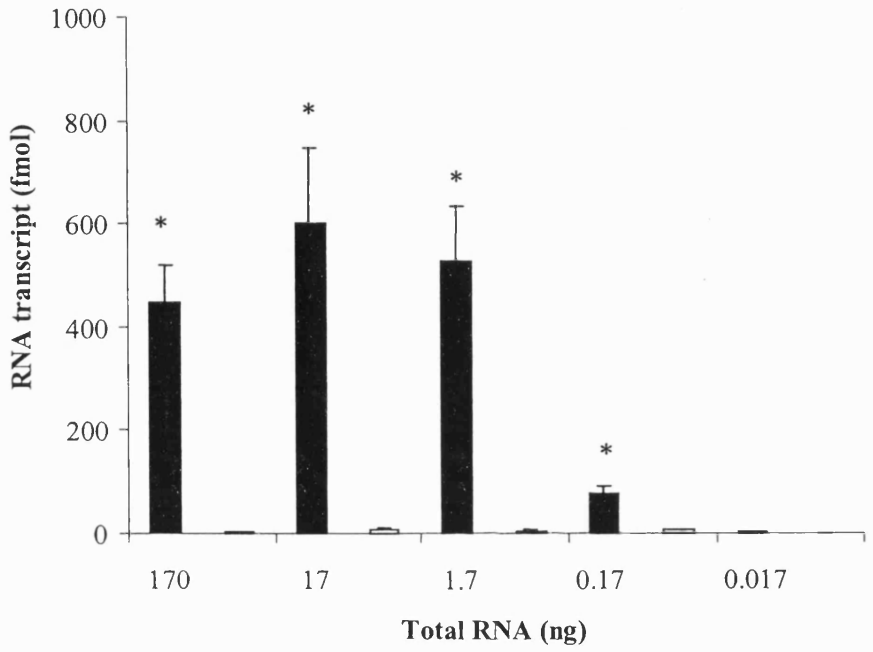


* $P \leq 0.05$

3.3.4 SMART assay of 23S rRNA extracted from *P. aeruginosa* PAO1

Once the SMART assay was fully optimised for detection of a synthetic target, the assay was used to quantify 23S rRNA levels in *P. aeruginosa* PAO1. Total RNA was extracted from *P. aeruginosa* cultures grown for 16 h at 37°C in LB broth. The SMART assay was undertaken over a range of RNA concentrations in order to determine the minimum sensitivity achievable in experimental conditions. Fig. 3.6 shows that detectable levels of amplification of 23S rRNA could be achieved using from 170 ng down to 0.17 ng of total RNA. Background amplification from the negative controls was considerably lower than those seen with a synthetic target. These results indicated that the SMART assay could accurately detect 23S rRNA in *P. aeruginosa* total RNA.

Fig. 3.6 SMART assay of 23S rRNA extracted from *P. aeruginosa* PAO1. Cells were grown for 16 h in LB broth at 37°C. Total RNA was extracted and then diluted to a concentration range of 170 ng to 0.17 ng and assayed using SMART (black bars). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bars). n=3 ± SD.

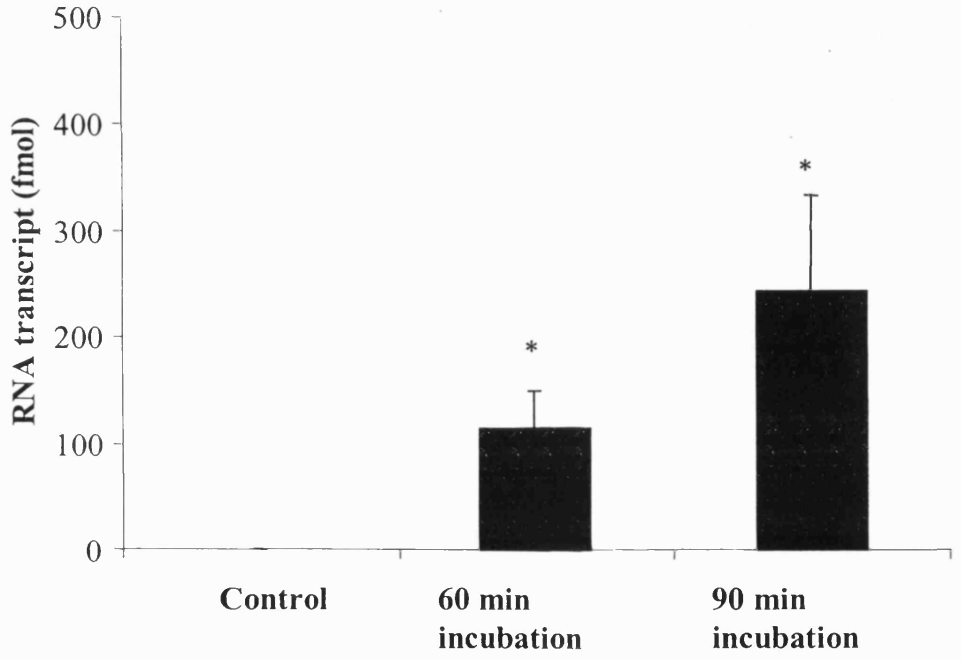


* P ≤ 0.05

3.3.5 Optimisation of SMART assay "One-Pot" method

The "One-Pot" version of the SMART assay was designed to reduce the assay to a single incubation at 41°C and incorporate the enzyme additions into one step. The assay was undertaken using *P. aeruginosa* 23S rRNA synthetic target. Research undertaken by Cytocell indicated that the amplification step should be optimally incubated at 41°C for either 60 or 90 min. Results shown in Fig. 3.7 show that 90 min incubation at 41°C provided a higher level of amplification than at 60 min. Although amplification levels produced by the "one-pot" method were lower than those of the standard assay, it was chosen to be used in future research as negative controls produced considerably lower background amplification and the overall assay time was reduced by 120 min.

Fig. 3.7 The effect of incubation time on SMART assay of a synthetic *P. aeruginosa* 23S rRNA target. SMART assay "one-pot method" was used to determine the effect of incubation time at 41°C on the amplification of 50 amol of *P. aeruginosa* synthetic 23S rRNA target (black bars). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bars). n=3 ± SD.



* $P \leq 0.05$

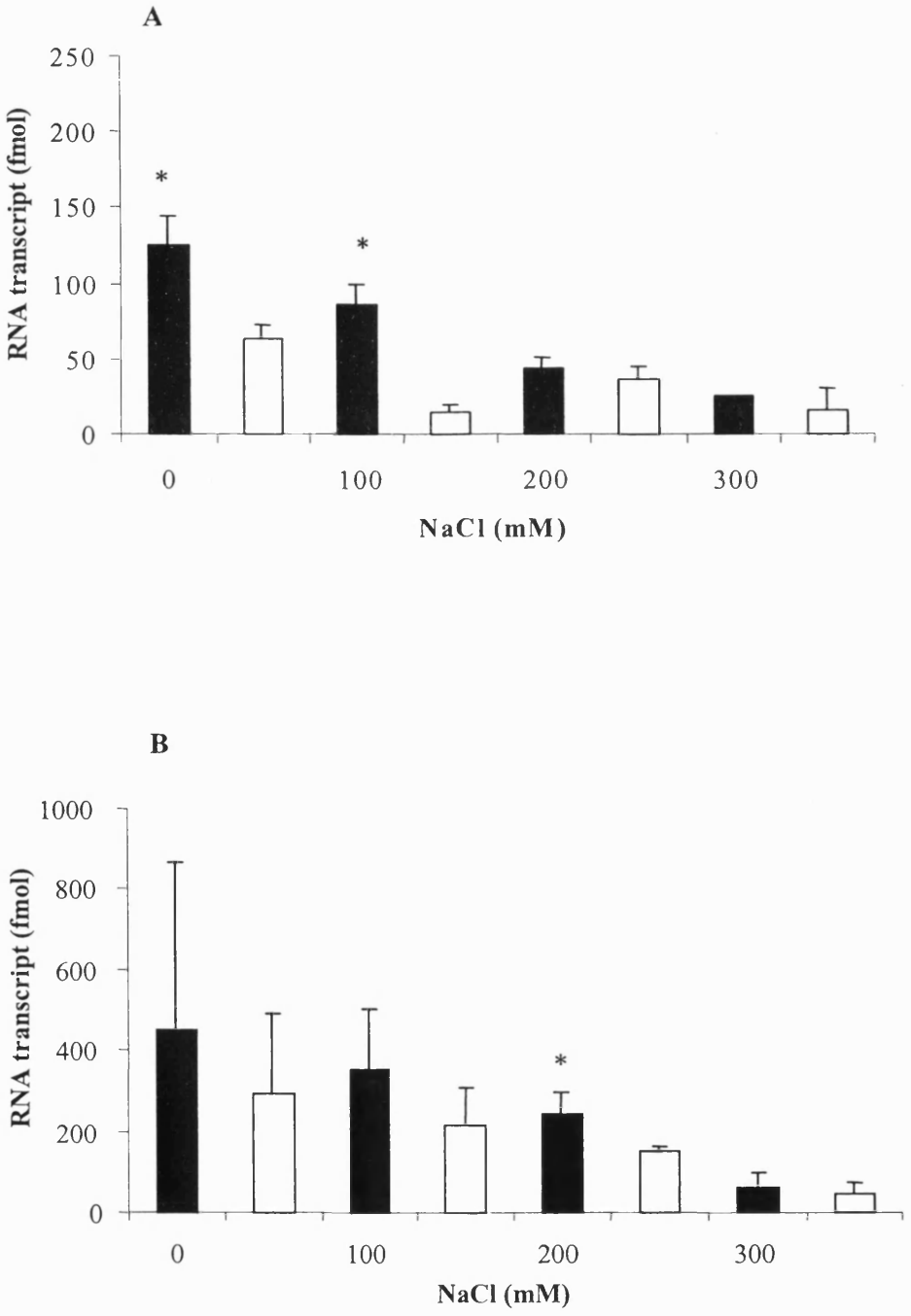
3.3.6 The SMART assay additional amplification step

The standard SMART assay consists of production of RNA-1 following formation of the 3WJ with the target sequence. This signal is further amplified using a linear amplification probe to produce the RNA-2 signal, which is then quantified by ELOSA. It was proposed that assay sensitivity could be increased by amplification of RNA-2 to produce a third RNA signal termed RNA-3. Consequently, an additional amplification probe for the RNA-2 was designed and the concentrations of the SMART reagents were increased to compensate for the additional step. The amplification of both target reactions and negative controls is highly salt-dependent and so the assay was conducted at a range of NaCl concentrations. To provide a positive control, the standard amplification assay was undertaken using the same reagent concentrations as the additional amplification assay.

Fig. 3.8 A. illustrates that the amplification levels in both target and control reactions in the standard assay were reduced with increasing NaCl concentration. At salt concentrations of 0 and 100 mM signal to noise values were approximately 2:1 and 5:1, respectively, whilst at 200 and 300 mM NaCl elevated background amplification reduced signal-to-noise values to 1:1. Results shown in Fig. 3.8 B. indicate that the additional amplification step provided an approximately four-fold increase in amplification levels in target reactions when compared with the standard assay. However, background amplification levels were also increased by at least four-fold in the additional amplification assay, resulting in signal-to-noise ratios of at least 1:1 in all reactions. Unfortunately, this indicated the overall sensitivity of the assay was not increased. Furthermore intra-assay variability was far higher in the additional amplification assay compared with the standard assay. Therefore it was concluded that the additional amplification assay would not be used in further studies.

Fig. 3.8 Comparison of the effects of additional amplification steps on the SMART assay of a synthetic *P. aeruginosa* 23S rRNA target. SMART assay of a 23S rRNA synthetic target was conducted incorporating **A.** a single amplification step and **B.** an additional amplification step at a range of NaCl concentrations (black bars). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bars). n=3 ± SD.

* P ≤0.05

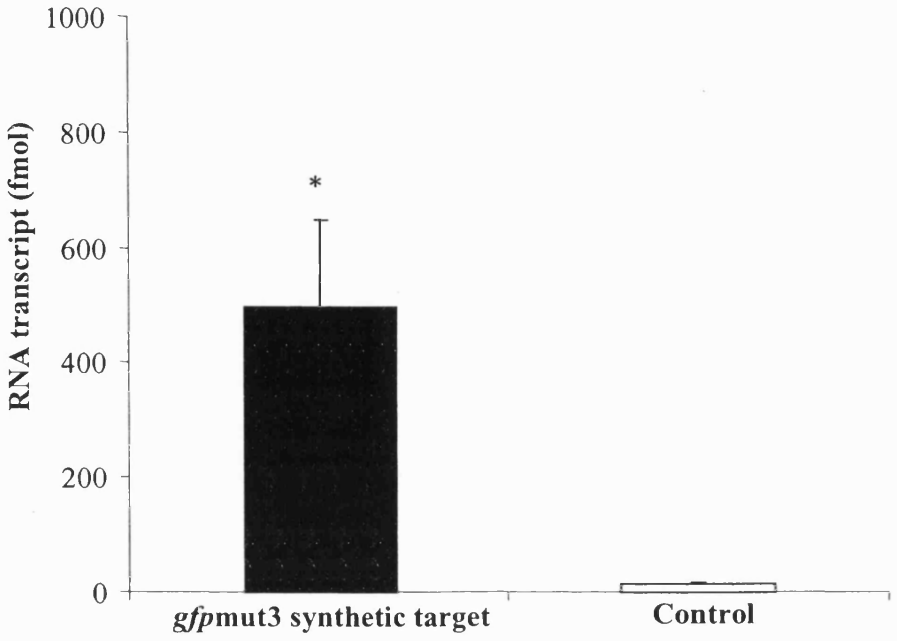


3.4 OPTIMISATION OF SMART ASSAY for *gfp*mut3 TARGET

3.4.1 SMART assay amplification step

The next stage of the study was to develop the technique for measuring the expression of *gfp* fusion strains. The SMART assay was first undertaken using a synthetic *gfp*mut3 target to ensure that probes designed for this junction were working reproducibly. Results shown in Fig. 3.9 indicate that 50 amol of target produced 450 fmol of RNA transcript, whilst negative controls produced 15 fmol of RNA transcript. Although target amplification levels were comparable with the SMART assay of the 23S rRNA, the 40:1 signal-to-noise ratio was significantly higher than that achievable for a 23S rRNA target.

Fig. 3.9 SMART assay of synthetic *gfpmut3* target. SMART assay of 50 amol of *gfp*mut*3* synthetic target (black bar). *M lysodeikticus* genomic DNA was used as a negative control (white bar). n=3 ± SD.

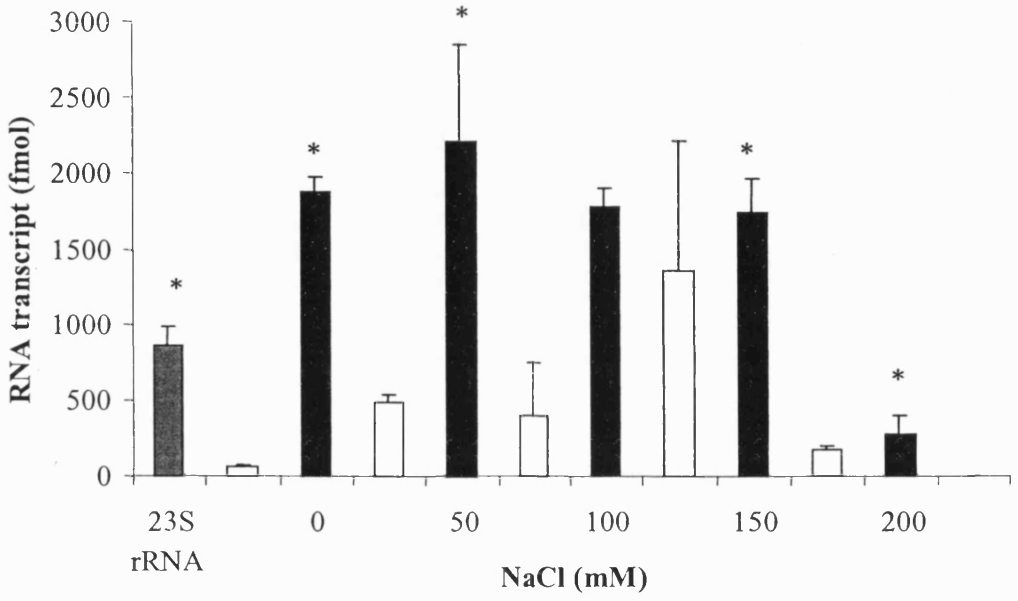


* P ≤0.05

3.4.2 Optimisation of salt concentration for SMART assay of *gfp*mut3 target

As described in section 3.3.3, optimal NaCl concentrations vary between different targets and therefore concentration range experiments for the SMART assay using *gfp*mut3 probes were performed. The SMART assay was undertaken at NaCl concentrations of 0, 50, 100, 150 and 200 mM. Fig. 3.10 shows that salt concentrations of 0, 50, 100 and 150 mM elicited high levels of amplification, producing approximately 2000 fmol of RNA transcript. The amplification generated by the negative controls was more salt-dependent; concentrations of 0 and 50 mM produced signal-to-noise ratios of 4:1, whilst 100 mM NaCl produced a ratio of less than 1:1. At a 200 mM NaCl concentration background amplification was very low but the amount of RNA transcript produced by the target reactions was very low. At a salt concentration of 150 mM, 280 fmol of RNA transcript was produced in the control reaction, which corresponded to a signal-to-noise ratio of 7:1. The high level of target amplification and low background amplification ensured a 150 mM concentration of NaCl was chosen for further work using the *gfp*mut3 probes.

Fig. 3.10 SMART assay of a synthetic *gfp*mut3 at a range of NaCl concentrations. SMART assay was undertaken at a range of salt concentrations, using 50 amol of *gfp*mut3 synthetic target probe (black bars). A positive control was provided by 50 amol of synthetic *P. aeruginosa* 23S rRNA target (grey bar). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bars). n=3 ± SD.

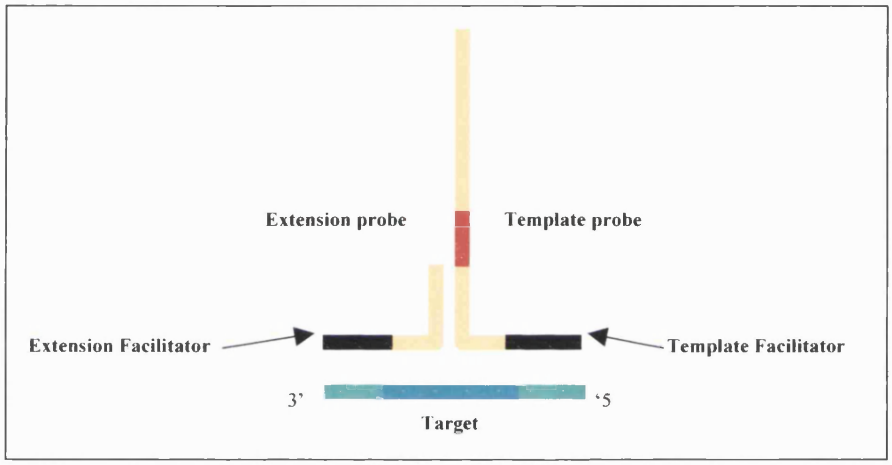


* P ≤ 0.05

3.4.3 The effect of facilitator probes on the sensitivity of the SMART assay

As illustrated in Fig. 3.11, facilitator probes are designed to anneal to target sequences immediately adjacent to the "foot" of either the template or extension probes. This increases the stability of the 3WJ complex and can improve target amplification and reduce background amplification.

Fig. 3.11 Annealing of facilitators. Facilitators are designed to anneal to the target immediately adjacent to the "feet" of the extension and template probes. They stablise the 3WJ which increases target-specific signal.



Results in Fig. 3.12 illustrate the effects of 200 amol of facilitator probes, on the amplification of both 50 and 100 amol of synthetic *gfp*mut3 target. The standard SMART assay using 100 amol of target produced 78 fmol of RNA transcript. The addition of extension facilitators increased amplification 2-fold, producing 143 fmol of RNA transcript. Whilst the addition of template facilitators increased amplification 2.5-fold, producing 183 fmol of RNA transcript. The addition of both facilitators to the assay did not increase amplification any higher than that achievable with the template facilitator alone. Negative control values did not substantially increase with the addition of either facilitator and hence the overall sensitivity of the SMART assay was improved. These results were mirrored by those achieved using

the lower 50 amol concentration of target probe and although use of facilitators did increase assay sensitivity the effects were less pronounced.

Increasing facilitator concentration to 500 fmol did not increase assay sensitivity any further. Fig. 3.13 illustrates that RNA transcript levels produced by 100 amol of target are approximately the same as those achieved at lower facilitator concentrations. However, addition of 500 fmol of extension facilitator produced 125 fmol of RNA transcript from 50 amol of synthetic target. Although significantly larger than the corresponding value achieved using 200 fmol of facilitator, the standard deviation values were very high and so overall results suggested that 200 fmol of template facilitator would increase the sensitivity of the SMART assay of *gfp*mut3 targets.

Fig. 3.12 The effect of 200 fmol facilitator probes on the SMART assay of a synthetic *gfp*mut3 target. The SMART assay of synthetic *gfp*mut3 target was conducted with 200 fmol of facilitator probes. Black and grey bars represent the amount of RNA transcript detected at synthetic *gfp*mut3 target concentrations of 50 and 100 amol respectively. *M. lysodeikticus* genomic DNA was used as a negative control (white bars). Facilitator probes were added to the assay as follows: A) No facilitator; B) facilitator for extension probe; C) facilitator for template probe D) facilitators for both template + extension probes. n=3 ± SD. All results were significant different from control values , $P \leq 0.05$

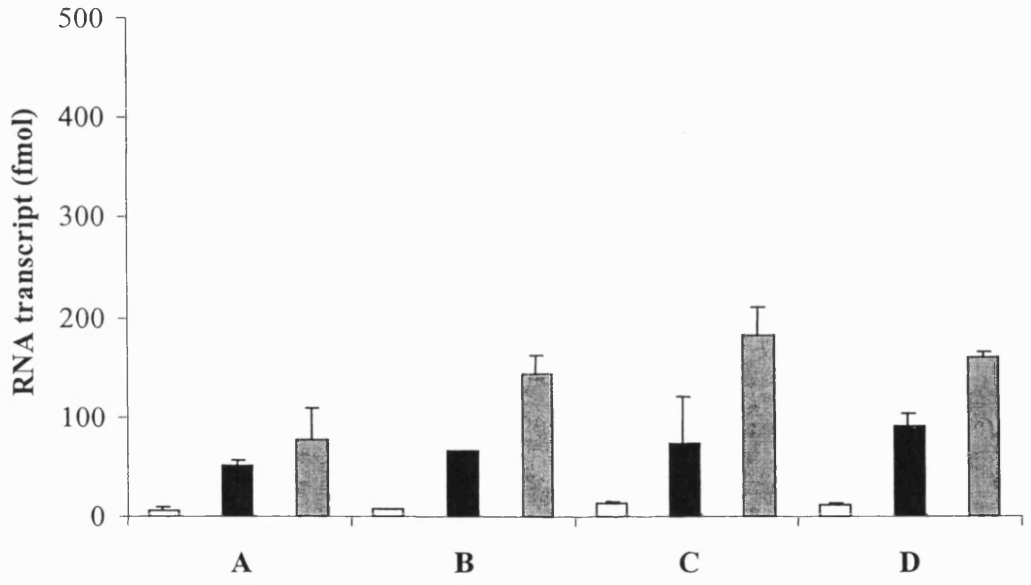
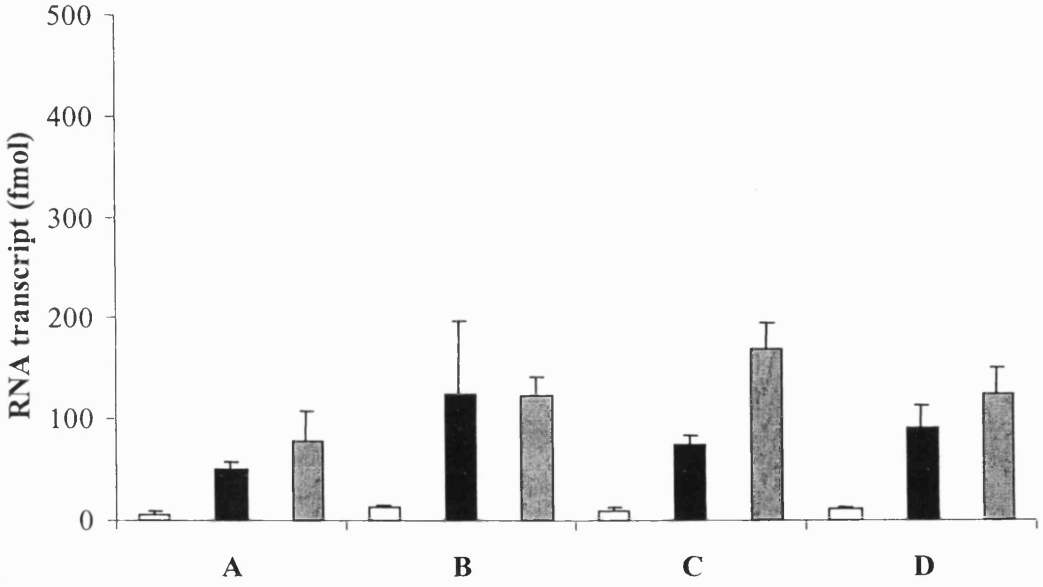


Fig. 3.13 The effect of 500 fmol facilitator probes on the SMART assay of a synthetic *gfp*mut3 target. The SMART assay of synthetic *gfp*mut3 target was conducted with 500 fmol of facilitator probes. Black and grey bars represent the amount of RNA transcript detected at synthetic *gfp*mut3 target concentrations of 50 and 100 amol respectively. *M. lysodeikticus* genomic DNA was used as a negative control (white bars). Facilitator probes were added to the assay as follows:
A) No facilitator; B) facilitator for extension probe; C) facilitator for template probe
D) facilitators for both template + extension probes. n=3 ± SD. All results were significant different from control values , $P \leq 0.05$



3.5 QUANTIFICATION OF *gfp*mut3 IN *P. aeruginosa* FUSION STRAINS

3.5.1 SMART assay of *gfp*mut3 fused to a constitutive plasmid promoter

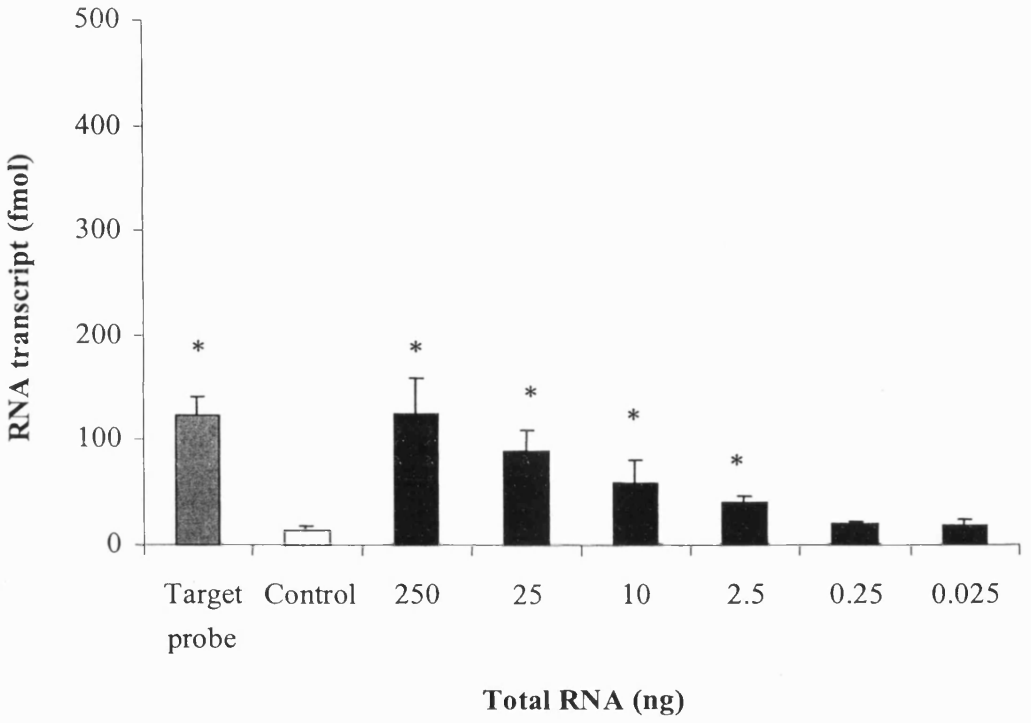
As the SMART assay had detected *gfp*mut3 mRNA using a synthetic target probe, the next stage was the detection of target sequence in total RNA samples extracted from *P. aeruginosa*. The high copy number plasmid pIAPX2, which contains a *gfp*mut3 sequence fused to a constitutive *lac* promoter, was transformed into *P. aeruginosa* PAO1. This fusion strain would produce high levels of *gfp*mut3 throughout the growth cycle and consequently sufficient levels of mRNA for detection by the SMART assay. Negative controls were provided by total RNA extracted from *P. aeruginosa* PAO1/pIA101, which contains the *gfp*mut3 sequence fused to a scrambled *lac* promoter. A synthetic *gfp*mut3 target probe was used as a positive control for the SMART assay, with *M. lysodeikticus* genomic DNA as the corresponding negative control. The levels of 23S rRNA were assayed in each total RNA sample to provide an additional positive control for RNA integrity.

Fig. 3.14 shows that target amplification of *gfp*mut3 could be detected from 250 ng down to 2.5 ng of *P. aeruginosa* PAO1/pIAPX2 total RNA. Background amplification in negative control reactions remained constant regardless of RNA concentration and so signal-to-noise ratios at target concentrations below 2.5 ng were below the minimum of 2:1, thus illustrating the minimum sensitivity of the SMART assay of *gfp*mut3 in this strain. The integrity of the assay was confirmed by synthetic target and negative control reactions producing 122 fmol and 15 fmol of RNA transcript respectively, corresponding to a signal-to-noise ratio of 10:1.

Results shown in Fig. 3.15 indicate that the SMART assay of 23S rRNA junction is more sensitive than the *gfp*mut3 junction, achieving satisfactory levels of amplification from 0.25 to 250 ng of total RNA. Even at 0.025 ng total RNA there was discrimination between the target and control results. However, the target amplification at higher target concentrations was reduced in comparison to those of the mid-range, with the highest amounts of RNA transcript produced at a 10 ng RNA concentration. The integrity of the assay was confirmed by the synthetic target and

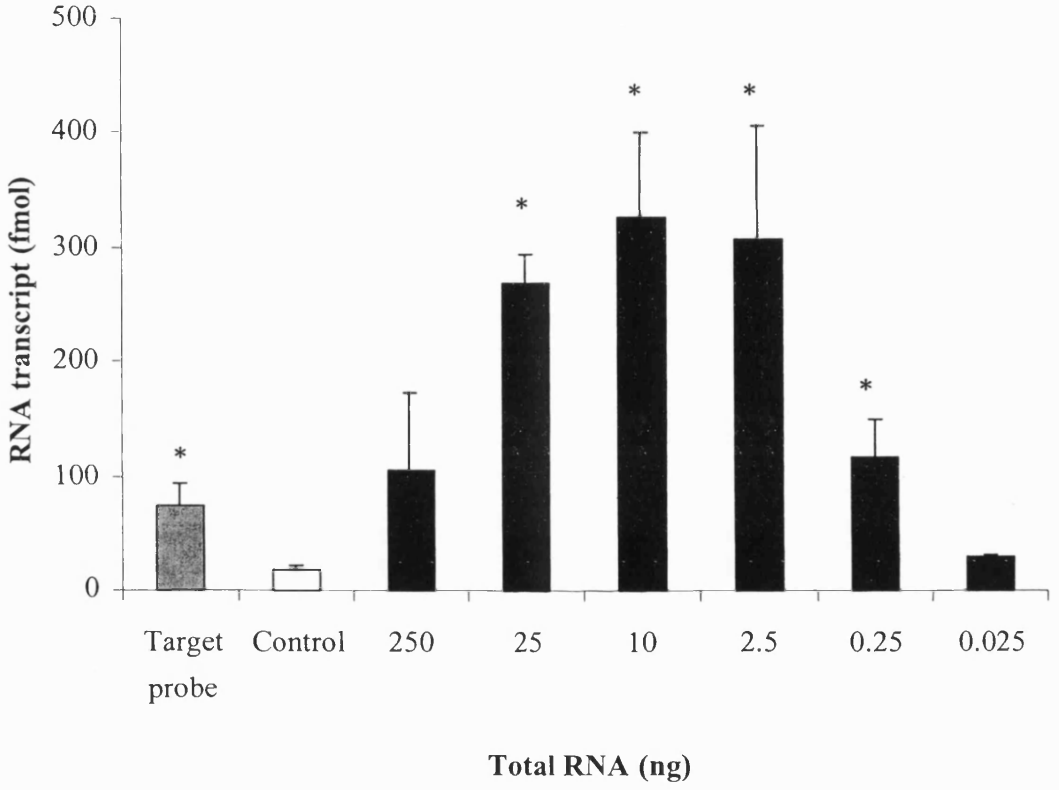
negative control producing 73 fmol and 18 fmol of RNA transcript, respectively, corresponding to a signal-to-noise ratio of 4:1.

Fig. 3.14 SMART assay of *gfp*mut3 mRNA in total RNA extracted from *P. aeruginosa* containing a high copy number, plasmid-borne *lac*::*gfp*mut3 fusion . SMART assay of *gfp*mut3 mRNA was conducted at a range of total RNA concentrations extracted from *P. aeruginosa* PAO1/pIAPX2 (black bars). A positive control was provided by 50 amol of synthetic *gfp*mut3 target (grey bar). *M. lysodeikticus* genomic DNA was used as a negative control (white bar). n=3 ± SD.



*P ≤0.05

Fig. 3.15 SMART assay of 23S rRNA in total RNA extracted from *P. aeruginosa* PAO1/ pIAPX2. SMART assay of 23S rRNA was conducted at a range of total RNA concentration extracted from *P. aeruginosa* PAO1/pIAPX2 (black bars). A positive control was provided by 50 amol of 23S rRNA synthetic target (grey bar). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bar). n=3 ± SD.



* P ≤0.05

3.5.2 SMART assay of *gfp*mut3 levels in a plasmid-borne *rpoS*/*gfp*mut3 fusion strains SS429 and SS431

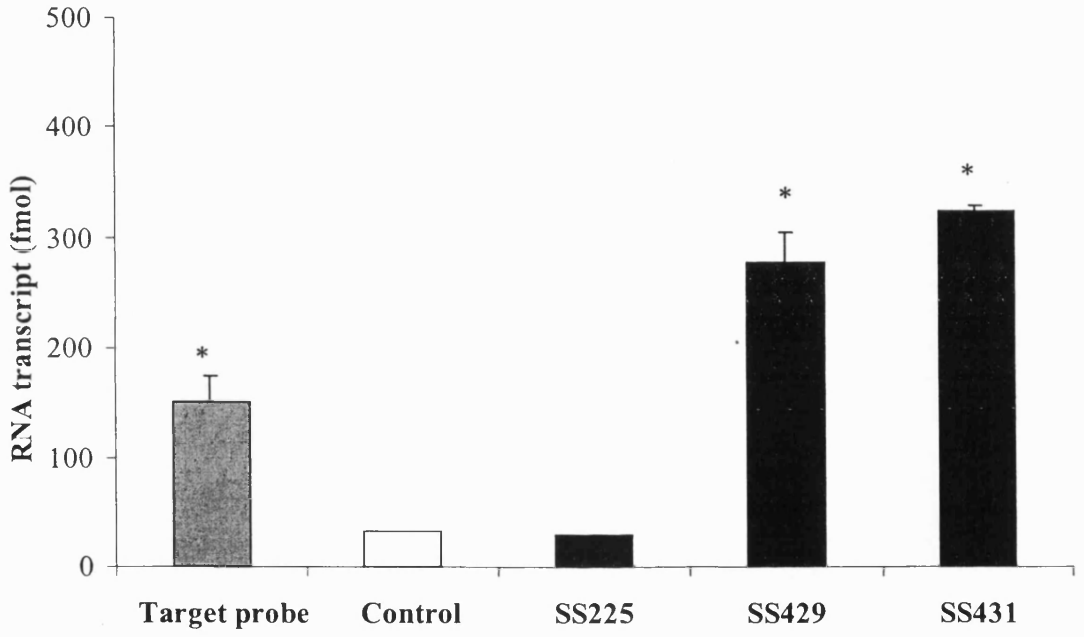
Optimisation of the SMART assay of *gfp*mut3 was undertaken to detect the expression of a range of genes without re-designing probes for each target sequence. The stationary phase sigma factor, *rpoS* is a global regulator of the stress response in *P. aeruginosa* and regulates the transcription of a range of virulence factors (see section 1.6). As other aspects of this study were investigating *rpoS* expression it was decided to establish if its expression in *P. aeruginosa* could be detected by the SMART assay using stationary phase cultures of *P. aeruginosa* containing plasmid-borne *rpoS*::*gfp*mut3 fusions. Two fusion strains were selected which contained a *gfp*mut3 sequence fused to the *rpoS* promoter on a high copy number plasmid; *P. aeruginosa* SS429 (*rpoS*::*gfp*mut3 translational fusion) and *P. aeruginosa* SS431 (*rpoS*::*gfp*mut3 transcriptional fusion) and the negative control was provided by *P. aeruginosa* SS225 which contains a blank copy of the pUCP20 vector used to construct these fusions.

Log-phase starter cultures of each strain were inoculated into fresh CDM_{10} complete and incubated at 37°C until early stationary phase. Total RNA was extracted using the Qiagen RNeasy kit. A synthetic *gfp*mut3 target probe was used as a positive control for the SMART assay and *M. lysodeikticus* genomic DNA as the corresponding negative control. The levels of 23S rRNA were assayed in each sample to provide an additional positive control for RNA integrity.

Fig 3.16 illustrates that high levels of *gfp*mut3 amplification were detected in both *P. aeruginosa* SS429 and SS431 RNA extracts, producing 277 and 324 fmol of RNA transcript respectively. Background amplification was very low, with SS225 producing 24 fmol of RNA transcript which corresponded to signal-to-noise values of at least 10:1 for both fusion strains. The SMART assay of the synthetic target probe produced sufficient levels of transcript to confirm the assay was working reproducibly. Fig 3.17 confirms the integrity of 23S rRNA in total RNA extracted

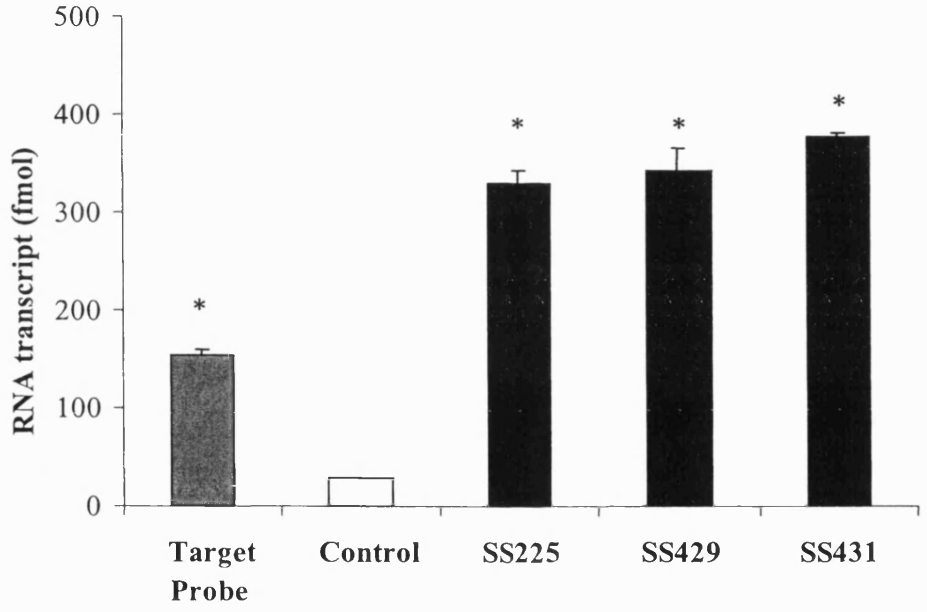
from of SS225, SS429 and SS431. These results illustrate that the SMART assay was sufficiently sensitive to detect *rpoS* expression using plasmid-borne *gfp* fusions.

Fig. 3.16 SMART assay of *gfp*mut3 levels in total RNA extracted from stationary phase cultures of plasmid-borne *rpoS/gfp*mut3 fusions SS429 and SS431. Cultures of *P. aeruginosa* SS225 (blank plasmid), SS429 (plasmid-borne *rpoS/gfp*mut3 transcriptional fusion) and SS431 (plasmid-borne *rpoS/gfp*mut3 translational fusion) were grown to stationary phase in CDM_{10} complete. SMART assay was used to determine *gfp*mut3 levels in 25 ng of total RNA extracted from relevant cultures (black bars). A positive control was provided by 50 amol of *gfp*mut3 synthetic target (grey bar). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bar). n=3 ± SD.



* $P \leq 0.05$

Fig. 3.17 SMART assay of 23S rRNA extracted from stationary phase cultures of plasmid-borne *rpoS/gfp*mut3 fusions SS429 and SS431. Cultures of *P. aeruginosa* SS225 (blank plasmid), SS429 (plasmid-borne *rpoS/gfp*mut3 transcriptional fusion) and SS431 (plasmid-borne *rpoS/gfp*mut3 translational fusion) were grown to stationary phase in CDM_{10} complete. SMART assay was used to confirm 23S rRNA integrity in 25 ng of total RNA extracted from relevant cultures (black bars). A positive control was provided by 50 amol of 23S rRNA synthetic target (grey bar). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bar). n=3 ± SD.



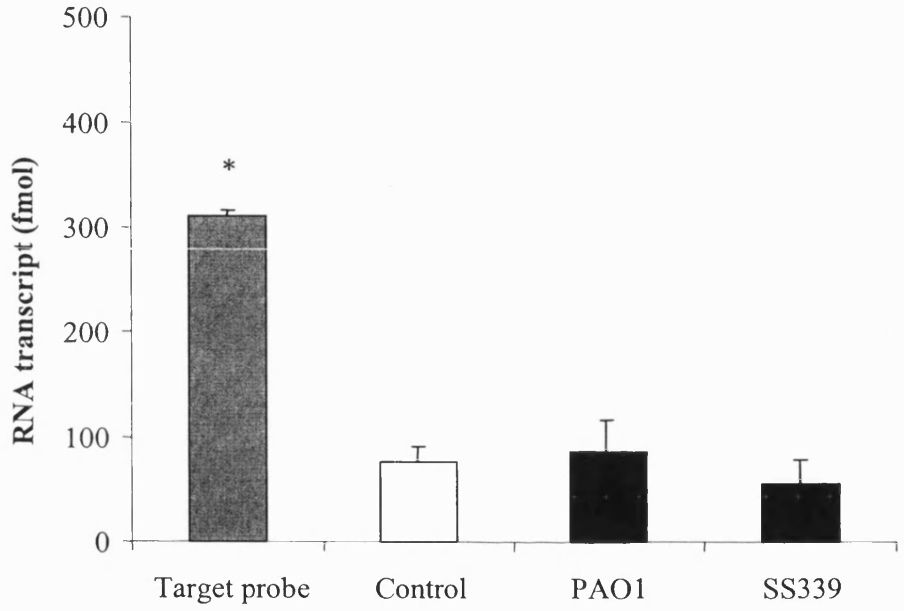
* $P \leq 0.05$

3.5.3 SMART assay of *gfp*mut3 levels of chromosomal *rpoS/gfp*mut3 fusion strain SS339

Although the SMART assay had been shown to detect of *rpoS/gfp*mut3 mRNA transcribed from a high copy number plasmid, the overall aim of the research was to determine if the SMART assay could be used to monitor gene expression at the chromosomal level. This could be achieved by extracting total RNA from cultures of the chromosomal *rpoS/gfpmut3* fusion strain, *P. aeruginosa* SS336 and quantifying mRNA levels using the SMART assay. The parental wild-type *P. aeruginosa* PAO1 was used as a negative control.

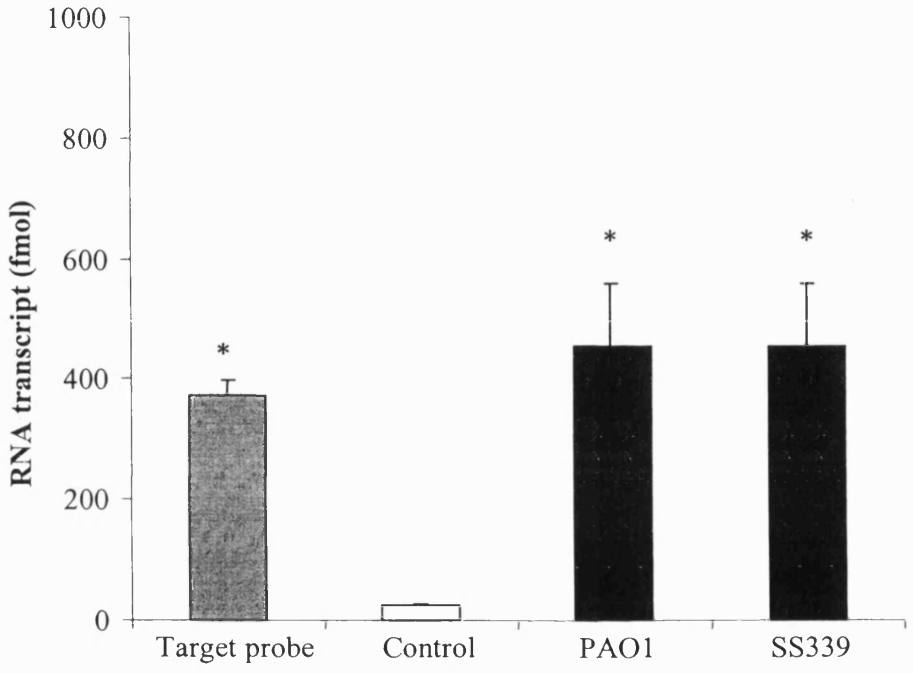
Results shown in Fig 3.18, indicate that the SMART assay was not sufficiently sensitive to detect *rpoS/gfpmut3* mRNA in 25 ng of total RNA extracted from stationary phase cultures of SS336, with RNA transcript levels approximately the same as those seen in the wild-type strain *P. aeruginosa* PAO1. These low levels of amplification were not due to assay error as the synthetic target probe was amplified to a sufficiently high level and the integrity of the total RNA was confirmed by high levels of 23S rRNA amplification in both the fusion strain and the wild type (Fig 3.19). These results suggest *rpoS/gfp*mut3 mRNA was not present in these samples or it was not in sufficient quantities to be detected by the SMART assay.

Fig 3.18 SMART assay of *gfp*mut3 levels in total RNA extracted from stationary phase cultures of *P. aeruginosa* PAO1 and chromosomal *rpoS*::*gfp*mut3 SS339. Cultures of *P. aeruginosa* PAO1 (control) and SS339 (chromosomal *rpoS*/*gfp*mut3 translational fusion) were grown to stationary phase in CDM_{10} complete. The SMART assay was used to determine *gfp*mut3 levels in 25 ng of total RNA extracted from relevant cultures (black bars). A positive control was provided by 50 amol of *gfp*mut3 synthetic target (grey bar). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bar). n=3 ± SD.



* $P \leq 0.05$

Fig. 3.19 SMART assay of 23S rRNA extracted from stationary phase cultures of *P. aeruginosa* PAO1 and SS339. SMART assay of 23S RNA levels in 25 ng of total RNA extracted from *P. aeruginosa* PAO1 and SS339. A positive control was provided by 50 amol of 23S rRNA synthetic target (grey bar). Background amplification was calculated using *M. lysodeikticus* genomic DNA as a negative control (white bar). n=3 ± SD.



3.6 DISCUSSION

The SMART assay was originally designed by Cytocell Ltd as a tool for rapid identification of pathogens for diagnostic purposes. Target-specific amplification and a single assay temperature suggested the potential of using SMART to quantify bacterial gene expression. The following results were obtained in this study:

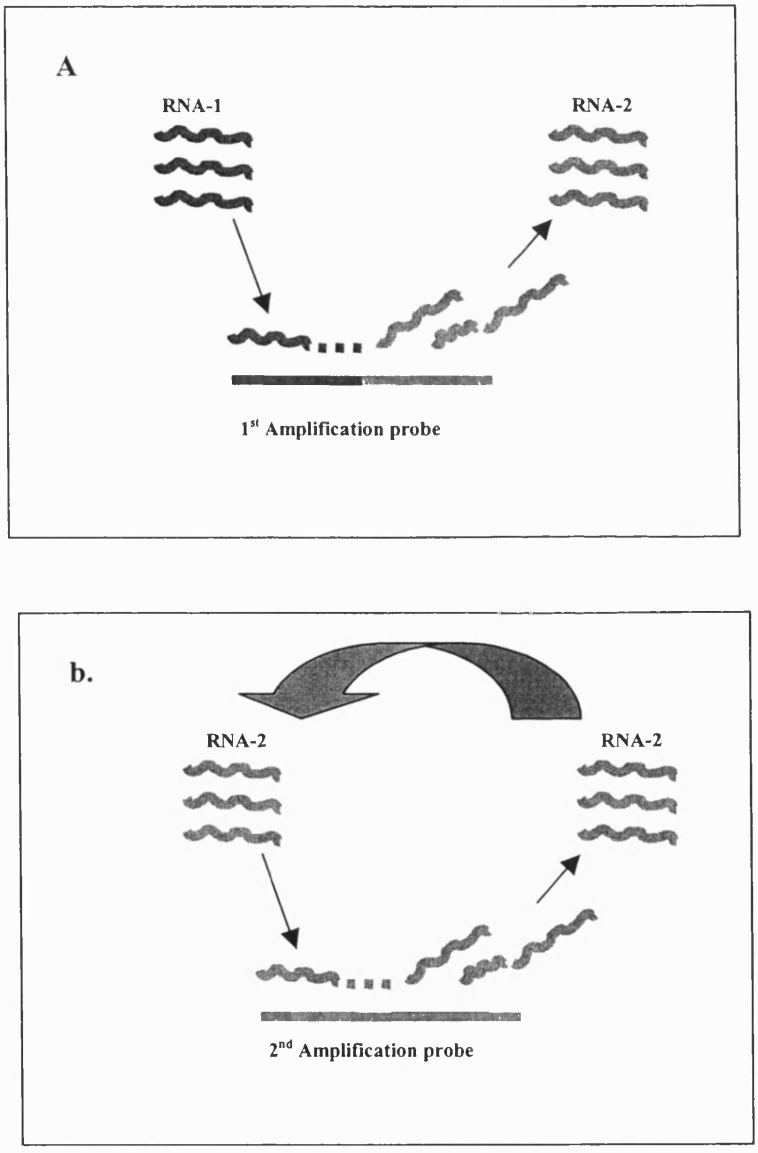
- Determination of experimental modifications which increased assay sensitivity
- The use of the SMART assay to quantify 23S rRNA levels in total RNA
- Quantification of *gfp*mut3 mRNA transcribed both from a constitutive plasmid and a plasmid-borne *rpoS*::*gfp*mut3 fusion in *P. aeruginosa* PAO1.

3.6.1 Additional amplification steps in the SMART assay

The SMART assay is based upon the formation of the 3WJ between the template and extension probes and target sequence. Conditions are optimised to ensure this complex is formed only in the presence of the target sequence and so new probes are designed for each individual target. The signal generated from the 3WJ is too small to be used as a sensitive marker of gene expression as it only provides an approximate 20-fold amplification of target signal. However, it does provide a point of comparison when optimising newly designed junctions. The amplification step of the SMART assay produces high levels of RNA-2 by amplifying the RNA-1 signal transcribed from the 3WJ complex. This step increased amplification 500-fold when detecting 23S rRNA and *gfp*mut3 target sequences. Although a further amplification step provided an additional 4-fold increase amplification, this was accompanied by a sizeable increase in background amplification and so did not improve assay sensitivity. Future work should continue to optimise this step as reducing in high levels of background amplification would provide a significant increase in assay sensitivity.

An alternative method of amplification could be employed by cycling the amplification of the RNA-2 signal. In the presence of a target sequence, he 3WJ is formed producing the RNA-1 signal which is then amplified by the amplification probe, producing the RNA-2 signal. A secondary amplification probe could be designed to amplify RNA-2 but instead of producing third RNA signal, more RNA-2 signal could be generated (see Fig. 3.20). This would again anneal to the amplification probe and so RNA-2 could be generated exponentially. This would provide an additional amplification of the RNA-2 signal without requiring any additional probes. Background noise should be approximately at the levels obtained using the standard protocol, which is far lower than those incurred in the additional amplification assay.

Fig. 3.20 Cycled amplification of RNA-2. SMART assay is conducted using the standard method with forming of the 3WJ in the presence of a target, which then generates the RNA-1 signal. **A**. RNA-1 anneals to the amplification probe which is extended and transcribed to produce RNA-2. **B**. RNA-2 then anneals to the additional amplification probe and instead of generating a third signal, more RNA-2 is produced, which can in turn anneal and generate even more signal.



3.6.2 Background amplification in the SMART assay

Background amplification or non-specific production of RNA signals is generated primarily in the amplification steps of the SMART assay. This is reflected in the degree of background noise generated in each step of the assay; minimal levels in the 3WJ step, increased levels in the amplification step and high levels in the additional amplification step. Background amplification may be due either to the non-specific annealing of template and extension probes or to non-specific transcription from the single-stranded template probe of which both would result in the production of RNA-1 in the absence of a target sequence (Wharam *et al.* 2001). Although, this can be minimised by altering assay conditions such as probe and salt concentration, high background amplification remains a problem for improving assay sensitivity.

Due to the nature of the SMART assay, non-specific annealing between template and extension probes independently of target sequence is an obvious cause of background amplification. However, in this situation levels detected in the amplification steps would be comparable with those achieved in the 3WJ step. This was not reflected in the results, which showed a sharp increase in background noise in each subsequent amplification step, until a point is reached where, regardless of salt concentration, signal-to-noise ratios were less than 2:1, reflecting no discrimination between the target amplification and background amplification. Therefore, the most plausible explanation for background amplification is non-specific transcription of single-stranded probes (template probe, amplification probe and secondary amplification probe) by T7 RNA polymerase (T7 RNAP). Although this enzyme requires a double-stranded promoter sequence to initiate transcription (Maslak and Martin 1993), low level "leaky" transcription can occur from a single-stranded sequence (Kukarin *et al.* 2003). Consequently, RNA signals can be transcribed in the absence of the appropriate signal and this would be compounded with each additional amplification step of the assay and this would explain the increased production of signal in control reactions.

However, T7 RNAP still remains the best choice of RNA polymerase for the SMART assay for the following reasons. Firstly, the SMART assay requires a DNA polymerase to elongate the extension probe forming a double stranded promoter, from which T7 RNAP generates a RNA signal and *Bst* DNA polymerase and T7 RNA polymerase have already been confirmed as the best combination of enzymes in the current assay conditions (Wharam *et al.* 2001). Secondly, T7 RNAP, in contrast to most RNA polymerases, is able to carry out transcription in the absence of additional protein factors and requires a relatively short promoter sequence (Kochetkov *et al.* 1998). Both of these characteristics are essential for the SMART assay to be conducted efficiently and outweigh the negative aspects of this enzyme involving its role in the generation of background amplification.

Another potential cause of background amplification in the SMART assay is the actual temperature the assay is conducted at. Initially 41°C was selected as previous research had determined that it presented the best compromise for the optimal activity of both T7-RNAP and *Bst* DNA polymerase and consequently the assay itself. A potential drawback to this temperature is that it is at least 15°C lower than the melting temperatures of the assay probes which presents an increased chance of dimerisation, self-annealing and other non-specific annealing of the probes. The SMART assay is very dependent upon the activity of both polymerases but they lack the thermostability that would permit an increase in the assay temperature. Therefore, the use of a more thermostable enzymes such as *Taq* polymerase would allow the reaction temperature to be increased to a level that may reduce non-specific annealing and subsequently the background amplification, providing an overall increase in assay sensitivity.

3.6.3 The effect of facilitators on assay sensitivity

The sensitivity of the SMART assay has been shown to be highly dependent upon the stability of the 3WJ complex (Leontis *et al.* 1991) and initial SMART research used modifications in probe design to increase stability. This involved the incorporation of hexaethylene glycol linkers into the template probe at the junction site, which increased amplification and reduced background transcription from the single stranded template in the absence of target (Wharam *et al.* 2001). Therefore, any modification which improves the annealing of template and extension probes to the target sequence could potentially increase assay sensitivity, as shown by the use of facilitators in this study with extension and template facilitators increasing amplification levels by a factor of 2-fold and 2.5-fold respectively. This confirmed that assay sensitivity could be improved by increasing the stability of the 3WJ complex. Furthermore, the stability of the 3WJ was more dependent upon the template probe than the extension probe as demonstrated by the larger increase in amplification using template facilitators. This confirmed that template facilitators could be routinely used in the SMART assay of *gfp*mut3 to increase assay sensitivity.

3.6.4 SMART assay of 23S rRNA

The initial aim of the research was to determine the minimum sensitivity of the SMART assay for a 23S rRNA target, which could be used both as a positive control and as a model system for assay development. The SMART assay detected 117 fmol RNA transcript from as little as 0.25 ng of *P. aeruginosa* PAO1 total RNA. This minimum sensitivity is comparable to levels obtained using a 23S rRNA target in *E. coli* K12, from which 120 fmol of RNA transcript was detected from 0.1 ng of total RNA (Hall *et al.* 2002). Furthermore, it was reported that 23S rRNA target signals can be reproducibly detected from *E. coli* crude cell extracts (down to 10^4 cells per assay) (Wharam *et al.* 2001). This approach warrants further investigation in *P. aeruginosa* as RNA extraction methods are predominantly expensive, time consuming and complex (Rivas *et al.* 2001). The removal of the RNA extraction step would decrease processing time, minimise cell stress and increase the number of samples that could be routinely assayed.

Ribosomal RNA (16S and 23S) comprise almost 90% of total cellular RNA and are present in approximately equal amounts (Apirion and Miczak 1993) and log-phase cells in planktonic culture can contain up to 70000 ribosomes (Andersen *et al.* 2001). Hence, RNA extracted from high density cultures should contain large amounts of rRNA which should be easily detected by the SMART assay. However, results suggested that higher concentrations of total RNA actually reduced the efficiency of the SMART assay, with target concentrations above 10 ng providing lower levels of amplification than concentrations below 10 ng. As the stability of the 3WJ has a direct influence on the sensitivity of the assay (Leontis *et al.* 1991), this effect could be due to the physical quantity of RNA either reducing the stability of the 3WJ complex by preventing the probes annealing to the target sequence or affecting the binding of enzymes to the probes. The activity of both DNA and RNA polymerases are dependent upon the appropriate alignment of the holoenzyme and the target sequence which, if affected, can reduce the efficiency of the enzymes and consequently the formation of any product (Lewin 1994). Therefore, any disruption of enzyme activity would inhibit polymerase-dependent steps of the SMART assay such as elongation of the extension probe by DNA polymerase and production of the RNA signals via T7 RNA polymerase.

3.6.5 SMART assay of *gfp*mut3

The main aim of this study was to determine the suitability of the SMART assay for monitoring gene expression in *P. aeruginosa* by using the assay to detect *gfp*mut3 mRNA from a) a plasmid fusion under the control of a constitutive *lac* promoter, b) a plasmid fusion under the control of a *rpoS* promoter and c) a chromosomal fusion under the control of an *rpoS* promoter. The use of a high copy number plasmid containing *gfp*mut3 fused to a constitutive *lac* promoter would provide large amounts of target mRNA which initially helped optimise assay conditions. Results showed that SMART could detect *gfp*mut3 in as a little as 2.5 ng of total RNA extracted from this fusion strain and also confirmed that SMART could be used to detect mRNA from bacterial cells. Probe sensitivity can differ significantly between targets and at least three different sets of probe are designed for each individual target, with the most sensitive being finally selected. It is difficult to directly compare the minimum

sensitivity of the 23S rRNA assay with that of *gfp*mut3 and although results indicated that the 23S rRNA assay was 10 times more sensitive than *gfp*mut3 and it should be noted that in 25 ng of total RNA from a fusion strain, the level of *gfp*mut3 mRNA generated from a high copy number plasmid would still be far lower than the concentration of 23S rRNA present in the cells. Therefore the sensitivity of the 23S rRNA and *gfp*mut3 can only be directly compared at exactly the same target concentrations but due to the difficulties of directly isolating and quantifying mRNA any direct comparisons of sensitivity are currently unachievable.

By far the widest selection of *gfp*mut3 fusion strains has been constructed in plasmids whose high copy number, ease of construction and ability to shuttle fusions between strains has made them an invaluable molecular tool. This study showed that the SMART assay could accurately quantify expression by detecting mRNA transcribed from *gfp*mut3 fusion under the control of an *rpoS* promoter, which is up-regulated in *P. aeruginosa* as cells enter stationary phase (Tanaka and Takahashi 1994). This confirmed that SMART could be used to monitor the expression of genes under the control of cellular factors which are upregulated in response to certain stimuli. However, the influence of high-copy number in *gfp* fusions should not be overlooked, as leaky transcription from plasmid promoters and the effects of high levels of fusion protein on cellular metabolism have not been fully addressed, there remains considerable debate regarding the extent to which plasmid fusions accurately reflect bacterial gene expression.

The expression of chromosomal fusions is more reflective of gene expression at the cellular level and so cells are less susceptible to the effects of overexpression of the fusion protein. Unfortunately, the SMART assay was unable to detect target mRNA in a chromosomal *rpoS*/*gfp*mut3 fusion although expression of the fusion protein was confirmed by direct fluorescence measurements (see chapter 5). Even though the SMART assay was not sufficiently sensitive to detect target mRNA in 25 ng of total RNA, conducting the assay at higher concentrations of total RNA could perhaps permit some level of quantification, as total RNA samples were extracted from just 1 ml of stationary phase culture. However, mRNA is rapidly degraded, present at low

concentrations and difficult to isolate using standard extraction methods. Consequently, higher total RNA concentrations would have to be used to permit detection using the SMART assay. However, as previously discussed, high quantities of rRNA appear to reduce the efficiency of the SMART assay and so any increase in signal produced by the chromosomal fusions targets may be inhibited by the elevated levels of 23S rRNA. Overall, this indicates that increasing target concentration would not be a practical way of using SMART to detect chromosomal fusion expression, especially as other techniques such as RT PCR and LightCycler PCR could quite adequately detect mRNA in the samples assayed (Freeman *et al.* 1999).

3.6.6 Applications of the SMART assay

The primary use of GFP as research tool is the visualization of gene expression at a cellular level using fluorescence microscopy and expression in these cells can be quantified by using techniques such as FACS (Cormack *et al.* 1996). However, there has been little direct application of quantitative techniques to support the results achieved using microscopy. The SMART assay has been shown to detect target sequences reproducibly in both *E. coli* and cyanophage lysates (Wharam *et al.* 2001), (Hall *et al.* 2002) which suggest that the assay is not significantly inhibited by bacterial proteins and nucleic acids. It is also unaffected by components of growth media, unlike PCR which is affected by a wide selection of biological and experimental compounds (Wilson 1997). The ability to quantify target in cell lysates and a simple, yet reproducible methodology (compared with techniques such as FACS) suggests that once assay sensitivity is improved, SMART could be easily applied as a quantitative technique to support qualitative methods such as fluorescence microscopy.

The "foot" sequences of the template and extension probes that anneal to the target nucleic acid are the only variable sequences in the SMART assay. With all the other components such as the amplification and ELOSA probes being generic. This means almost any target could be detected with a minimum number of alterations to the assay. Furthermore, mutations in the chromophore of GFP have produced a variety of fusions with high sequence homology which can fluoresce over a wide range of

wavelengths (Tsien 1998). SMART probes were designed to anneal to target sequences outside the region associated with the GFP chromophore which ensured that assay probes were generic for as many *gfp* mutants as possible. This would allow the quantification of an even wider selection of fusions using the *gfp*mut3 probes and assay conditions described in this study.

This study demonstrated that the SMART assay could detect expression of target mRNA under the control of both a constitutive and inducible promoters suggesting its potential as a developmental tool in the design of plasmid fusions, where it could provide a semi-quantifiable measurement of promoter efficiency and target copy number. Although lacking the sensitivity of PCR, the main advantage of SMART as a quantification technique is the reproducibility of amplification levels between experiments. This contrasts with PCR, where even with stringent controls in place it is prone to highly variable levels of amplification, with even the most minor tube-to-tube variations resulting in major differences in levels of product (Freeman *et al.* 1999).

In conclusion, if the SMART assay is to be used as a quantitative technique the highest priority must be given improving assay sensitivity and this study showed that increases could be achieved with relatively minor alterations in assay conditions. Once able to detect mRNA at levels produced by low copy number targets such as chromosomal fusions, the SMART assay would be an ideal technique for comparing the relative efficiencies of a selection of promoters and could also could be employed for rapid and accurate measurement of gene expression.

4 IRON AVAILABILITY IN CDM_{10} COMPLETE AND ITS EFFECTS ON THE IRON STATUS OF *P. aeruginosa* PAO1

4.1 INTRODUCTION

The availability of iron is a limiting factor in the spread of any microbial community and bacteria commonly colonise environments containing a paucity of available iron. Several acquisition systems are employed by bacteria to obtain iron including chelation, reduction and low-affinity transport systems (reviewed Guerinot 1994). The most common method is secretion of low molecular weight, high-affinity chelators termed siderophores (Neilands 1973). *P. aeruginosa* produces two distinct siderophores in response to iron limited conditions: pyochelin (Sak *et al.* 1989) and pyoverdine (Meyer and Abdallah 1978). These proteins are secreted into the external environment to chelate ferric iron in response to iron-limited conditions and uptake of the siderophore-iron complex is regulated via membrane-bound receptors which are responsible for transport across the outer membrane of the cell (Cox 1980), (Redly and Poole 2003). These membrane receptors, termed iron-regulated outer membrane proteins (IROMPs) are highly expressed under iron-limited conditions and consequently their presence is used as an indication of iron starvation in a bacterial population. The outer membrane profile of *P. aeruginosa* cells can be visualized using SDS-PAGE, with IROMPs being identified as bands of approximately 80 to 90 KDa (Meyer *et al.* 1979).

Other research in this study will use the fluorescence of *rpoS*::*gfp*mut3 fusions to investigate the effects of nutrient limitation on expression of *rpoS* in planktonic and biofilm culture. The use of nutrients at a limiting concentration in chemically defined media (CDM) permits investigation of the response of micro-organisms to specific starvations. In particular, iron is difficult to control. The nature of iron in solution is highly dependent upon its ligand environment and any change in pH, buffer or chelator will affect its solubility and availability (Tadolini 1987a) and so the response of any organism to a starvation can be due to limiting concentrations of both the "chosen" nutrient and iron. This becomes especially important when investigating

the expression of genes involved in the stress response. It is more likely that *in vivo* environments would present multiple nutrient stresses for *P. aeruginosa*, however, as previously discussed, specific starvations can regulate the expression of a wide variety of genes and therefore the effects of individual starvations must be investigated before the effects of multiple stresses are investigated. Previous studies have used SDS-PAGE to identify an iron-starved phenotype in *P. aeruginosa* but this has been using cultures grown M9 minimal medium (Meyer *et al.* 1979) (Harding and Royt 1990). However, no such research has been conducted using CDM^{10}, which is a preferential medium as it permits a wider selection of nutrient starvations and MOPS has a higher buffering capacity than phosphate. Consequently, the presence of an iron-replete phenotype in CDM would have to be confirmed to ensure that further studies into starvation responses and *rpoS* expression were attributable solely to given nutrient and that CDM_{10} complete is a truly defined medium.

The aims of this research were:

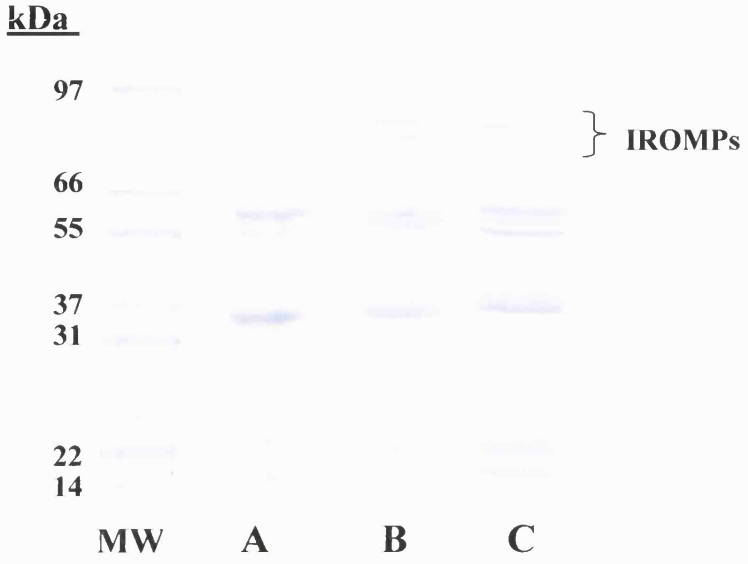
- To confirm an iron-replete phenotype for *P. aeruginosa* grown in CDM_{10} complete,
- If necessary, modify the growth medium to ensure an iron-replete phenotype is achieved,
- To confirm cells grown under a variety of starvations in planktonic and biofilm culture were not limiting for iron so the CDM could be used to investigate the effects of nutrient starvation on *rpoS* expression in *P. aeruginosa.*

4.2 DETERMINATION OF SUITABLE GROWTH MEDIA FOR CONTROL EXPRESSION OF IROMPs

To investigate the possibility of iron limitation in CDM_{10} complete it was necessary to identify growth conditions which provided positive and negative controls for expression of IROMPs in *P. aeruginosa* planktonic culture. LB broth is a complex and hence undefined medium with a pH of approximately 7.4 This medium contains sufficient iron levels to support growth and so cells grown in LB display little or no expression of IROMPs (Redly and Poole 2003). CDM_{10} complete is a defined medium containing 10 μM iron in which nutrients are in sufficient quantity for a culture to reach a theoretical maximum OD of 10. Therefore, cells grown in CDM with no added iron rapidly become limiting for iron and express high levels of IROMPs.

Results show that cells grown in LB broth displayed no expression of IROMPs (Fig. 4.1; lane A) which confirmed that this medium contained sufficient levels of iron to support growth and so cells grown in LB would provide a suitable example of a iron-replete phenotype. Conversely, cells grown in CDM with no added iron displayed strong IROMP expression, with distinct bands noted in the 66 to 97 kDa range (Fig 4.1; lane B) indicating that cells grown in CDM with no added iron express an iron-limited phenotype. Surprisingly, cells grown in CDM_{10} complete containing 10 μM iron also expressed IROMPs in the 66 to 97 kDa range (Fig 4.1; lane C). This suggested that cells grown in this "complete" medium were in fact limiting for iron and CDM_{10} complete would have to be modified to ensure an iron-replete phenotype could be achieved in this defined minimal medium

Fig. 4.1 Outer membrane protein profiles for *P. aeruginosa* PAO1. 10% SDS polyacrylamide gel electrophoresis of outer membranes isolated from *P. aeruginosa* PAO1 cells grown at 37°C in: (A) LB broth (B) CDM with no added iron and (C) CDM_{10} complete containing 10 μM iron.



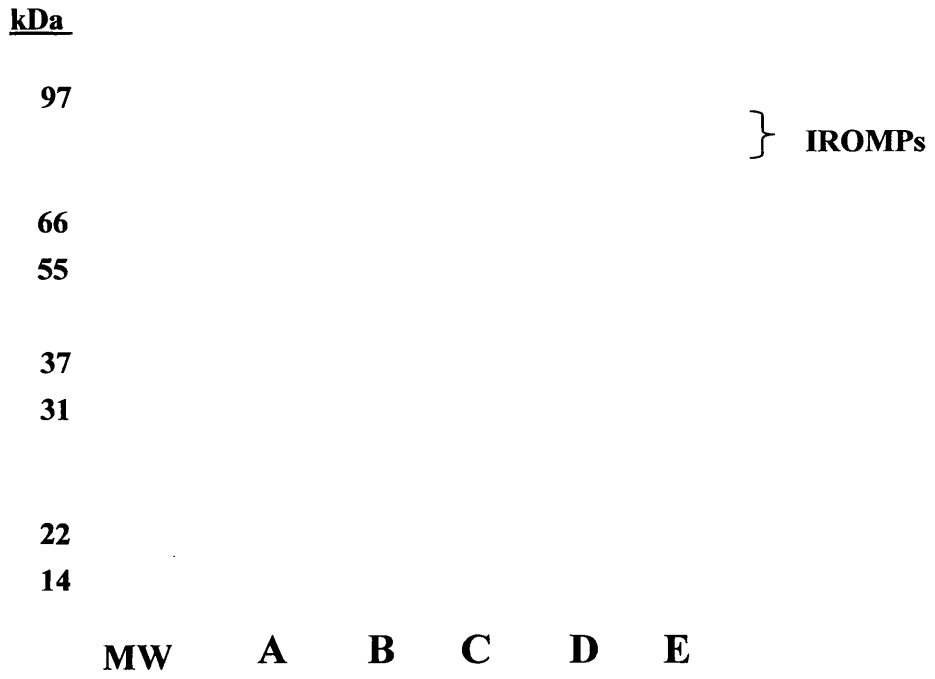
4.3 THE EFFECT OF IRON CONCENTRATION ON THE EXPRESSION OF IROMPs IN PLANKTONIC CULTURE

Reduced iron availability in planktonic culture has three possible causes. Firstly, the molar quantity of iron in the medium is not sufficient to support growth, which can be simply rectified by the addition of a higher concentration. Secondly, some aspect of the growth medium potentiates the oxidation of ferrous iron into its insoluble, ferric form. Finally, direct sequestration by some constituent (s) of the medium results in insufficient amounts of iron available to the bacteria

The presence of IROMPs in cultures grown in CDM_{10} complete indicated that the population was limiting for iron. However, the 10 μM concentration of iron added to the medium was in excess of the 6 μM requirement of *P. aeruginosa* (Brown *et al.* 1995). This suggested the molar quantity of iron was sufficient, but its availability was reduced by oxidation and/or sequestration. By increasing the concentration of ferrous iron added to the medium, the degree of oxidation/sequestration could be surpassed, providing sufficient available iron to support growth.

Cultures of *P. aeruginosa* PAO1 were grown in CDM at a range of iron concentrations and the expression of IROMPs was investigated. As illustrated previously (see Fig 4.1; lane C) cells grown in CDM_{10} complete containing 10 μM iron (Fig. 4.2; lane C) expressed IROMPs at levels comparable to growth in CDM with no added iron (Fig. 4.2; lane B). This confirmed that the addition of 10 μM iron to CDM failed to provide sufficient levels of available iron for the growth of *P. aeruginosa* PAO1. Results also showed that the addition of 100 μM iron (Fig. 4.2; lane D) and 200 μM iron (Fig. 4.2; lane E) to the CDM also produced high expression of IROMPs. Although the molar iron concentration of the CDM theoretically sufficient to support growth, the effects of reduced availability in the medium could not be overcome by increasing the concentration of added iron. Consequently, other aspects of the medium would have to be adjusted to ensure sufficient iron was available to the bacteria.

Fig 4.2 The effect of growth medium iron concentration on the outer membrane protein profile of *P. aeruginosa* PAO1. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown at 37°C, in (A) LB (B) CDM, no added iron; (C) CDM, 10 μM iron; (D) CDM, 100 μM iron; (E) CDM, 200 μM iron.

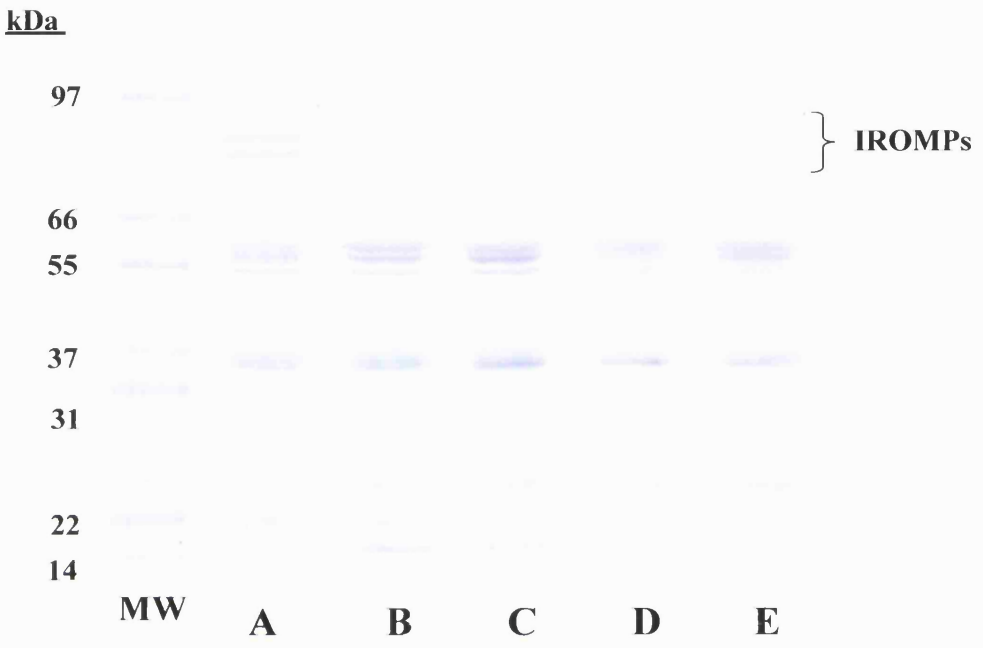


4.4 THE EFFECT OF GROWTH MEDIUM pH ON THE EXPRESSION OF IROMPs

The equilibrium between the ferrous and ferric oxidation states of iron in solution is dependent on many factors but is most influenced by ligand environment and pH. Iron autoxidation increases directly in proportion with the pH of the solution (Welch *et al.* 2003) and results indicated that iron limitation was probably due to insufficient solubility or availability. Therefore, a reduction in the pH of the growth medium could potentially reduce ferrous iron oxidation and hence increase iron availability. Cells were grown in CDM_{10} complete buffered to the following range of pH values: 7.0, 7.2, 7.4 and 7.6 and the expression of IROMPs were determined.

Results revealed that the changes in the pH of the growth medium did not affect the degree of iron limitation in the cells. Expression of IROMPs was noted at pH 7.0, 7.2, 7.4, and 7.6, (Fig. 4.3, lanes B to D) and furthermore the levels of expression appeared constant through this range. However, a different outer membrane protein profile was noted when using CDM with no added iron (Fig 4.3, lane A), which produced a higher band density in the IROMP region when compared to the other profiles. This suggesting that the iron limitation noted using CDM complete was not as pronounced as when cells were grown in CDM with no added iron.

Fig 4.3 The effect of growth medium pH on the outer membrane protein profile of *P. aeruginosa* PAO1. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown at 37°C, in (A) CDM with no added iron and CDM_{10} complete containing 10 μM iron, buffered to the following pH values: (B) 7.0; (C) 7.2; (D) 7.4 and (E) 7.6



4.5 THE EFFECT OF CITRATE ON THE EXPRESSION OF IROMPs

As results suggested that cells were becoming iron-limited due to the unavailability of ferrous (soluble) iron, it was thought the addition of a low-affinity iron chelator to the growth medium may increase bacterial iron-acquisition. *P. aeruginosa* possesses a distinct citrate transporter system which operates in a similar manner to siderophores. Citrate sequesters ferric iron present in the external medium and this complex is transported across the outer membrane of the bacterium via a membrane-bound transporter (Cox 1980). Cells were grown in LB broth and CDM_{10} complete containing 10 μM iron with and without the addition of 200 μM citrate. This would determine if citrate would reduce iron limitation in cells grown in CDM_{10} complete, with cells grown in LB providing a negative control for IROMP expression.

Comparison of cells grown in CDM with citrate (Fig. 4.4: lane D) and without citrate (Fig. 4.4; lane C) revealed similar expression of IROMPs, indicating that citrate was not reducing iron-limitation in the CDM. Interestingly, cells grown in LB containing 200 μM citrate (Fig. 4.4; lane B) displayed an additional band of approximately 40 kDa (note arrow), which was not expressed in either the LB control (Fig. 4.4; lane A) or the CDM cultures. Cells were also grown in CDM containing 10 μM iron and 200 μM citrate, at a range of pH values to determine if a reduction in pH improved the effects of citrate on bacterial iron acquisition. Cultures grown in CDM_{10} complete with and without added iron were used as controls. Results revealed that the addition of citrate at a range of pH values did not affect the iron-limitation in the population with IROMP expression noted through the range of pH values investigated (Fig. 4.5; lanes C to F) at levels comparable to cells grown in the absence of citrate (Fig. 4.5; lane A). This illustrated that at the investigated pH range, citrate failed to increase iron availability in the CDM

The inability of citrate to reduce iron limitation in CDM may be because, as a low-affinity chelator it is not as effective as an iron acquisition system in these conditions compared with siderophores such as pyoverdine. Alternatively, as *P. aeruginosa* can use citrate as a carbon source, it may be metabolised preferentially

over glucose, leaving insufficient levels of citrate to transport iron into the cells. Consequently, a weak acid chelator, which is not metabolised by *P. aeruginosa*, may be more successful in reducing iron limitation in the CDM.

Fig 4.4 The effect of citrate on the outer membrane protein profile of *P. aeruginosa* PAO1. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown at 37°C, in the following media: (A) LB; (B) LB + 200 μM citrate; (C) CDM_{10} complete; (D) CDM + 200 μM citrate.

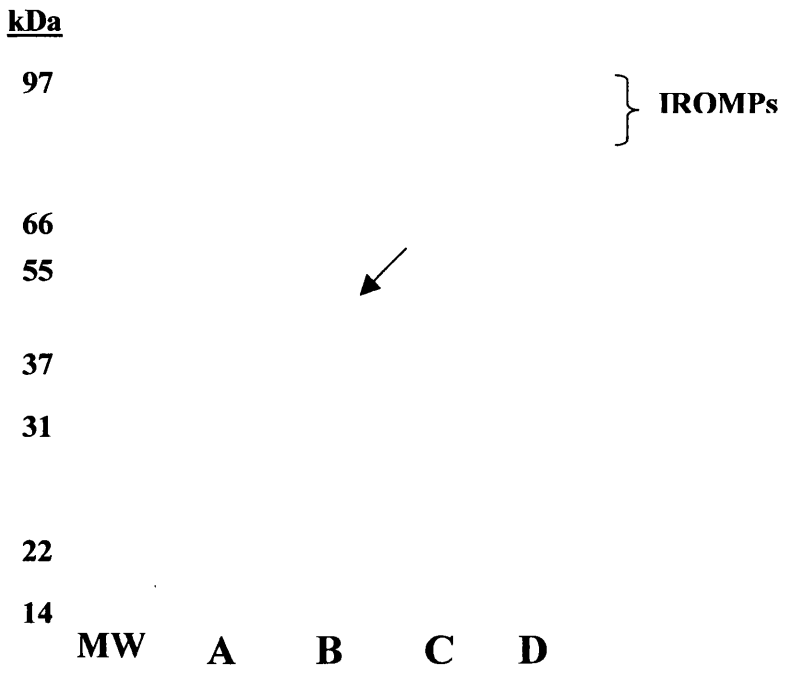


Fig 4.5 The effect of citrate on outer membrane protein profiles of *P. aeruginosa* PAO1 over a range of pH values. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown at 37°C, in: (A) CDM_{10} complete; (B) CDM with no added iron; (C) CDM + 200 μM citrate, pH 7.0 (D) CDM + 200 μM citrate, pH 7.2; (E) CDM + 200 μM citrate, pH 7.4; (F) CDM + 200 μM citrate, pH 7.6.

4.6 THE EFFECT OF ASCORBATE ON THE EXPRESSION OF IROMPs

It was decided to use ascorbate, another low-affinity chelator, to reduce iron-limitation in CDM. Ascorbate has a comparable chelating ability to citrate but cannot be used as a carbon source by *P. aeruginosa*. Therefore, cells were grown in LB broth and CDM_{10} complete containing 10 μM iron, with and without the addition of 200 μM ascorbate

Results indicate cells grown in CDM with 10 μM iron and 200 μM ascorbate (Fig. 4.6; lane D) displayed the same level of IROMP expression as cells grown in CDM with no added ascorbate (Fig. 4.6; lane C). This confirmed that ascorbate did not reduce the degree of iron-limitation in cells grown in CDM. A comparison of cells grown in LB (Fig. 4.6; lane A) and LB with 200 μM ascorbate (Fig 4.6, lane B) revealed no discernible difference in the expression profiles, indicating that *P. aeruginosa* did not express any additional outer membrane proteins in response to ascorbate in the growth medium.

Cells were also grown in CDM containing 10 μM iron and 200 μM ascorbate, at a range of pH values to determine if a reduction in pH improved the effects of ascorbate on bacterial iron acquisition. Cultures grown in CDM_{10} complete with and without added iron were used as controls. Results revealed that the addition of ascorbate at a range of pH values did not affect the iron limitation of the population. IROMP expression was noted through the range of pH values investigated (Fig. 4.7; lanes C to F) at levels comparable with cells grown in the absence of ascorbate (Fig. 4.7; lane A). This illustrated that at the investigated pH range, ascorbate failed to increase iron availability in the CDM.

Fig 4.6 The effect of ascorbate on the outer membrane protein profile of *P. aeruginosa* PAO1. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown at 37°C, in the following media: (A) LB; (B) LB + 200 μM ascorbate; (C) CDM_{10} complete (D) CDM + 200 μM ascorbate.

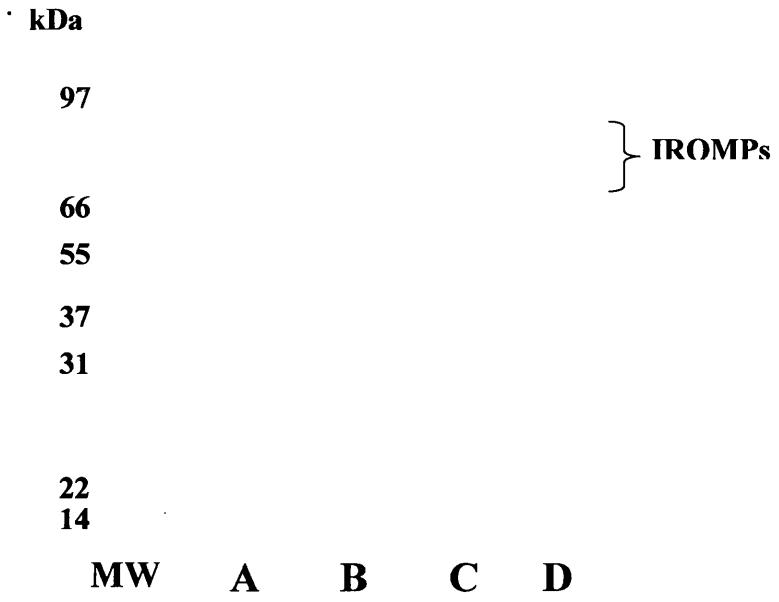
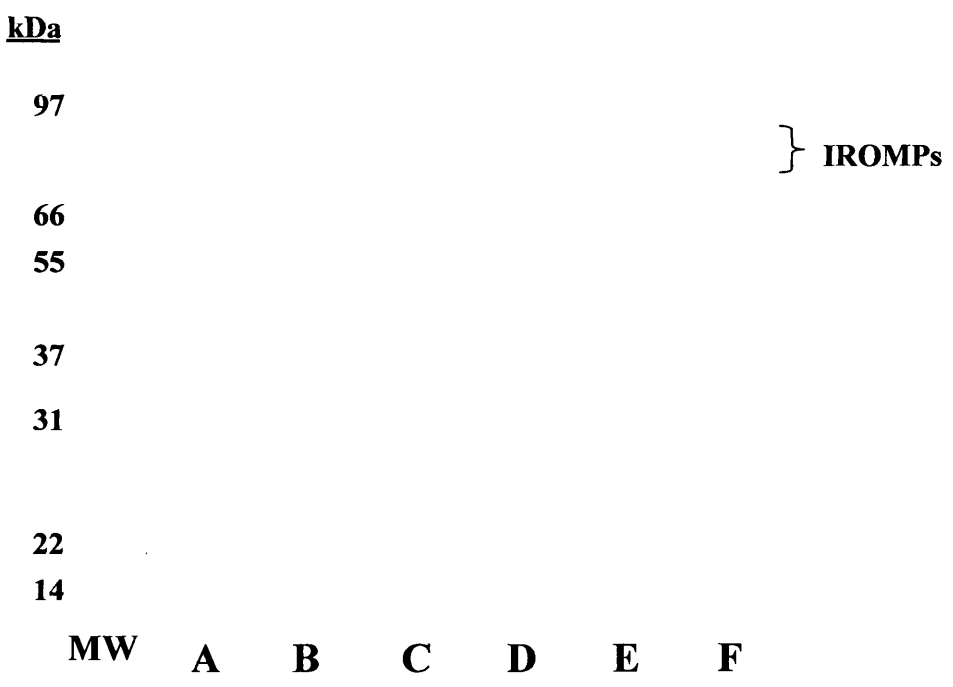


Fig 4.7 The effect of ascorbate on the outer membrane profile of *P. aeruginosa* PAO1 over a range of pH values. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown at 37°C, in: (A) CDM_{10} complete; (B) CDM with no added iron; (C) CDM + 200 μM citrate, pH 7.0 (D) CDM + 200 μM citrate, pH 7.2; (E) CDM + 200 μM citrate, pH 7.4; (F) CDM + 200 μM citrate, pH 7.6.



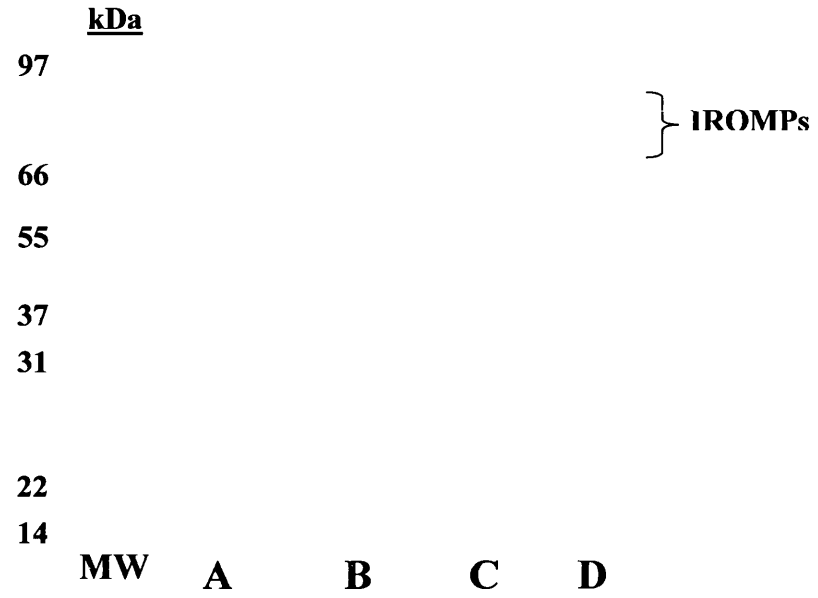
4.7 A COMPARISON OF THE EFFECTS OF MOPSO AND PHOSPHATE BUFFERED CDM ON THE EXPRESSION OF IROMPs

Previous studies have reported that minimal expression of IROMPs can be achieved using a chemically defined media such as M9 minimal medium, (Meyer *et al.* 1979) (Harding and Royt 1990). This in contrast to the current investigations which noted high levels of IROMP expression in cells grown in CDM_{10} complete at a variety of iron concentrations (Fig. 4.2). A comparison of medium components indicated that besides minor variations in concentration, the only clear difference between the M9 minimal medium and CDM_{10} complete was buffer composition. M9 medium employs a phosphate buffer system whilst CDM_{10} complete utilises MOPS or MOPSO buffer. The effect of these buffers in iron-limited and iron-replete conditions was investigated to determine their influence on iron availability in planktonic culture.

As observed in previously (Fig. 4.1), cells grown in CDM containing 10 μM iron buffered by MOPSO expressed IROMPs (Fig. 4.8; lane A). However, cells grown in CDM using phosphate buffer demonstrated minimal expression of proteins in the IROMP range, (Fig. 4.8, lane C) indicating that cells grown in phosphate buffered CDM were not limiting for iron. Cells grown in CDM containing phosphate buffer and no added iron, expressed high levels of proteins in the IROMP region (Fig. 1.8, lane D), confirming cells were iron limited.

These results suggest that MOPSO buffer decreased the availability of iron in CDM_{10} complete even at high concentrations, resulting in an iron-limited population. The replacement of MOPSO with phosphate buffer ensured minimal expression of IROMPs, indicating that sufficient iron was available to support growth without the de-repression of high affinity iron uptake systems. Consequently, results show that both an iron-replete and iron-limited phenotype could be achieved by growing cultures in CDM containing 100 μM phosphate buffer.

Fig 4.8 The effects of CDM buffer composition on outer membrane protein profiles of *P. aeruginosa* PAO1. 10% SDS polyacrylamide gel electrophoresis of outer membranes from *P. aeruginosa* PAO1 cells grown in CDM_{10} containing: (A) MOPSO buffer with 10 μM iron; (B) MOPSO buffer with no added iron; (C) phosphate buffer with 10 μM iron; (D) phosphate buffer with no added iron.



4.8 THE EFFECT OF IRON LIMITATION ON PYOVERDINE PRODUCTION IN *P. aeruginosa* CELLS GROWN IN MOPSO AND PHOSPHATE BUFFERS

As well as detection of IROMPs in the outer membrane protein fraction, iron limitation can also be confirmed by the spectrophotometric measurement of pyoverdine in culture supernatants. To confirm the differences in iron availability between MOPSO and phosphate-buffered CDM, cells were grown in both media containing either 10 μM iron or no added iron. The absorbance of cell-free supernatants were then measured at 402 nm to detect pyoverdine (Meyer and Abdallah 1978).

Table 4.1 confirms that cells grown in MOPSO-buffered CDM containing 10 μM iron were in fact limiting for iron, producing pyoverdine concentrations of 0.18 μM. Cells grown in MOPSO-buffered CDM no added iron provided very high levels of pyoverdine, producing 5.8 μM of pyoverdine, almost 31-fold higher than MOPSO-buffered CDM_{10} complete. Cells grown in phosphate-buffered CDM with 10 μM iron produced much lower levels of pyoverdine (0.04 μM). Cells grown in phosphate-buffered CDM with no added iron produced 1.1 μM of pyoverdine, almost 27-fold higher than the iron-replete conditions. Overall pyoverdine levels were almost 4-fold higher when the medium was buffered with MOPSO compared to phosphate. This supports the results obtained using SDS-PAGE that indicated that cells grown in a supposedly iron replete MOPSO-buffered medium were in fact limiting for iron.

Table 4.1 The effects of buffer composition on pyoverdine production in *P. aeruginosa* PAO1 grown in CDM with and without added iron. Molar concentration of pyoverdine was calculated as follows: absorbance at 405 nm/molar extinction coefficient for pyoverdine (1.9×10^4 $M^{-1}cm^{-1}$).

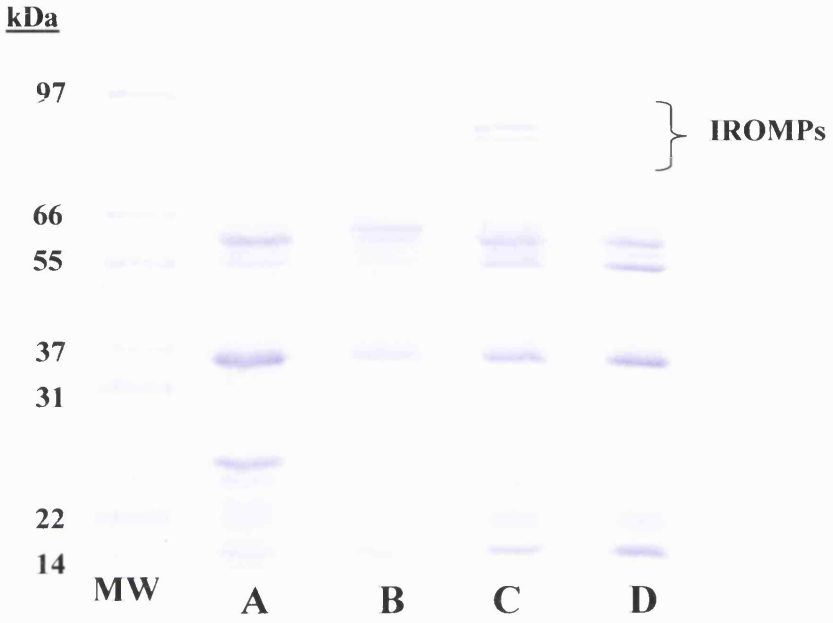
	Pyoverdine concentration (μM)	
	No added iron	10 μM added iron
MOPSO buffer	5.8	0.18
Phosphate buffer	1.1	0.04

4.9 CONFIRMATION OF IRON-REPLETE PHENOTYPE IN PLANKTONIC CULTURES GROWN IN NUTRIENT-LIMITED CDM

To investigate the effects of nutrient starvation in *P. aeruginosa*, cells were grown in CDM containing limiting concentrations of a specific nutrient. If results achieved using this media are to be attributed solely to the specific starvation and not iron limitation, the outer membrane profiles of cultures under a range of starvations would have to be analysed by SDS-PAGE to confirm that no IROMPs were expressed. Cells were grown in the following phosphate-buffered media: CDM_{10} Complete, CDM with 10 mM glucose, CDM with 15 μM magnesium sulphate (all containing 10 μM iron) and CDM with no added iron.

Results shown in Fig 4.9 indicate no expression of IROMPs in cells grown in CDM_{10} complete (lane A), glucose-limited CDM (lane B), magnesium-limited CDM (lane D) confirming that cells grown in these media were not limiting for iron, whilst cells grown in CDM with no added iron (lane C) displayed high levels of IROMP expression.

Fig 4.9 Confirmation of iron-replete phenotype in nutrient-limited planktonic culture. 10% SDS polyacrylamide gel electrophoresis of outer membranes from planktonic *P. aeruginosa* PAO1 cultures grown in (A) CDM_{10} complete with 10 μM iron; (B) glucose-limited CDM with 10 μM iron; (C) CDM with no added iron and (D) magnesium-limited CDM with 10 μM iron .

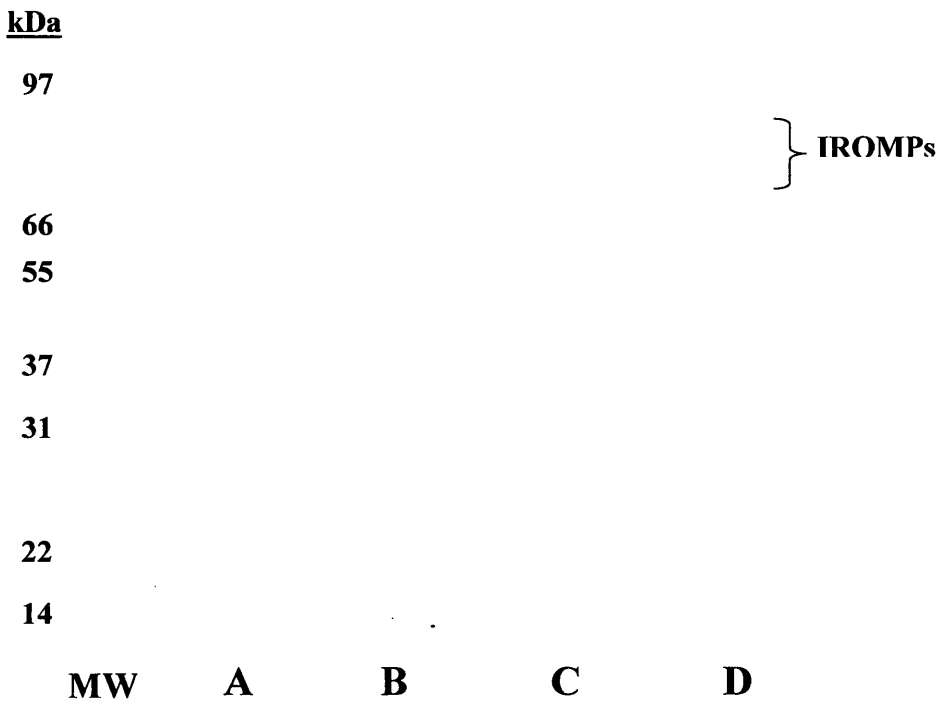


4.10 CONFIRMATION OF IRON-REPLETE PHENOTYPE IN BIOFILM CULTURES GROWN IN NUTRIENT-LIMITED CDM

As the effects of nutrient starvation in biofilm culture were also to be investigated it was decided to confirm that iron-replete phenotype could be achieved in biofilm cultures grown on CDM agar containing limiting concentrations of various nutrients. Cells were grown on the following phosphate-buffered agar: CDM_{10} Complete, CDM with 20 mM glucose, CDM with 30 μM magnesium sulphate (all containing 10 μM iron) and CDM with no added iron.

Results shown in Fig 4.10 indicates no expression of IROMPs in cells grown on: CDM_{10} complete agar (lane A), glucose-limited CDM agar (lane B) or magnesium-limited CDM agar (lane C), thus confirming that cultures grown on these media were not limiting for iron. Cells grown in CDM with no added iron (lane D) displaying high IROMP expression, confirming that these biofilm cultures were limiting for iron.

Fig 4.10 Confirmation of iron-replete phenotype in nutrient-limited biofilm culture. 10% SDS polyacrylamide gel electrophoresis of outer membranes from planktonic *P. aeruginosa* PAO1 cultures grown on agar containing: (A) CDM_{10} complete with 10 μM iron; (B) glucose-limited CDM with 10 μM iron; (C) magnesium-limited CDM with 10 μM iron and (D) CDM with no added iron.



4.11 DISCUSSION

It was necessary to determine if an iron-replete phenotype could be achievable in cells grown in CDM_{10} complete. If this was not possible, modifications were to be undertaken to ensure a truly defined medium could be used for nutrient limitation studies.

This study showed that:

- *P. aeruginosa* grown in CDM_{10} complete buffered with MOPSO had an iron limited phenotype, (indicated by expression of IROMPs) regardless of the concentration of iron added.
- An iron-replete phenotype could not be achieved either by reducing the medium pH to 7.0 or the by addition of weak acid chelators such as citrate and ascorbate.
- Replacement of MOPSO buffer with phosphate buffer established an iron-replete phenotype enabling CDM_{10} complete to be used for nutrient limitation studies.

4.11.1 Iron replete phenotype

As previously reported an iron-replete phenotype was confirmed in by minimal expression of IROMPs by *P. aeruginosa* PAO1 grown in LB broth (Meyer *et al.* 1979). The iron concentration of LB broth is approximately 30 μM (Wang *et al.* 1994) which is in excess of the 6 μM requirement of *P. aeruginosa* (Brown *et al.* 1995). However, a high media iron concentration in the medium does not guarantee an iron-replete phenotype, as demonstrated by IROMP expression in cells grown in CDM_{10} containing 10, 100 and 200 μM iron. It is unlikely that pH strongly influences the availability of the iron in LB broth as it is most commonly formulated at a slightly alkaline pH of approximately 7.4, which would favour insoluble, ferric iron. As LB broth is a complex growth medium, consisting of tryptone, yeast extract and salt, quantification of individual components is difficult. However, some component of this medium must ensure sufficient iron remains available to support bacterial growth.

4.11.2 Implications of iron limitation in MOPS-buffered medium

The use of a chemically defined medium allows investigation of the effects of a specific nutrient limitation on the bacterial phenotype. Initially CDM_{10} complete (containing 10 μM iron) was selected to confirm that any given response to a starvation was due solely to a single starvation and a not the multiple stress of the specific nutrient and iron. MOPS and MOPSO are widely used buffers (Tadolini 1987a) and was used in CDM_{10} complete, as it allows the investigation of phosphate limitation (which is clearly unachievable when using phosphate buffer). Surprisingly, cells grown in CDM_{10} complete containing 10 μM added iron expressed IROMPs at a level comparable to those grown with no added iron. This had important implications, as many previous studies, investigating a wide selection of targets have employed either MOPS or MOPSO to achieve buffer control in defined medium. This is of most consequence when using MOPS buffer when investigating nutrient starvation such as carbon (Suh *et al.* 1999) or carbon and phosphate (Jorgensen *et al.* 1999) in *P. aeruginosa*. The reduced iron availability in CDM_{10} complete with 10 μM iron, coupled with the high iron requirement of this bacterium (Brown *et al.* 1995), indicates that cultures grown in these studies were likely limiting for iron and consequently the results cannot be specifically ascribed to carbon or carbon/phosphate limitation but rather the effects of multiple starvations.

Williams *et al.* (1984) reported that *K. pneumoniae* grown in iron-limited conditions expressed IROMPs several generations before end of the exponential phase, suggesting that iron limitation in planktonic culture is detected and acquisition systems are upregulated before any reduction in growth rate is noted. This highlights to importance of confirming an iron-replete phenotype for each growth medium, which can be achieved by measuring markers of iron limitation such as IROMP expression (Meyer *et al.* 1979) siderophore production (Meyer and Abdallah 1978) or iron-dependent gene expression (Turner *et al.* 1995). This process was conducted for all growth media to be used for nutrient-limitation studies in chapter 5 and results confirmed minimal IROMP expression was achievable in both planktonic and biofilm cultures grown in CDM_{10} complete (phosphate buffered) as well as cells grown under glucose and magnesium limitation. This ensured that any data obtained were the

result of limiting concentrations of a specific nutrient and not iron-limited conditions in the growth media.

Although MOPS is used to buffer defined media for the growth of other bacteria, it may be less important if that species has a low iron requirement. For example, the effects of carbon starvation on the expression of *rpoS* in *E. coli* have been investigated using M9 minimal salts media, buffered by MOPS. However, as the molar iron requirement of *E. coli* is as low as 0.5 μM (Brown *et al.* 1995) it is unlikely that the MOPS would have such a dramatic effect as that seen in *P. aeruginosa*, whose iron requirement is over 10-fold higher. This is supported by a recent study that has detected no IROMP expression in both planktonic and biofilm cultures of *E. coli* grown in MOPS-buffered CDM_{10} complete with 10 μM added iron (P. Wood, personal communication).

4.11.3 Media pH and iron availability

The oxidation state of iron in solution is highly pH dependent, with the ferrous state being favoured in acidic conditions (Tadolini 1987a). However, most biological media are buffered to a pH of 7.0 and above, which favours insoluble, ferric iron. Therefore, the reduction the pH of CDM_{10} complete from pH 7.6 to pH 7 would potentially increase solubility but this modification failed to reverse iron-limitation in this medium. Experiments were conducted at a physiological pH range and consequently, reducing the pH of the medium to more acidic conditions would have far reaching consequences regarding the organism's response to low pH. Furthermore, CDM_{10} complete was selected to investigate stress responses and the expression of the general stress response regulator *rpoS* is reported to increase in response to low pH in both *P. aeruginosa* (Jorgensen *et al.* 1999) and *E. coli* (Small *et al.* 1994). However, the pH range selected was very narrow and perhaps unlikely to provide a significant change in iron availability, especially as acidic conditions were never appropriately investigated. A significant lowering of a pH range could be applicable in further work, especially as the ubiquitous nature of *P. aeruginosa* means that it is able to survive a broad range of environmental pH. This is especially relevant in the CF lung where the inflammation induced by *P. aeruginosa* results in a

acidic conditions. However, the aim of this study was to investigate the effect of specific nutrient starvation on *rpoS* expression and so reduction in medium pH should only be undertaken if acid stress would not directly effect the subject under investigation. The presence of chelators in a growth medium have a greater influence on iron availability than pH, with phosphate buffer (considered a strong chelator) promoting the autoxidation of ferrous iron even at pH 6.5 (Welch *et al.* 2003). This suggests that even if the pH of the growth medium is reduced to acidic values, the presence of chelating compounds within the medium can reduce iron availability to a greater degree.

4.11.4 Iron availability and weak acid chelators

The low-affinity chelators operate in the presence of high levels of environmental iron (Harding *et al.* 1990) and *P. aeruginosa* possesses a distinct ferric citrate transport system which citrate chelates ferric iron and the complex then enters the cell via a membrane-bound uptake mechanism (Cox 1980). Cells grown in LB broth containing 200μM citrate expressed a 40 kDa protein that was not present in cultures grown in LB only which corresponded to the ferric citrate receptor previously noted in *P. aeruginosa* (Cox 1980). However, IROMP expression was noted in cells grown in CDM_{10} complete with 200 μM citrate over a range of physiological pH values, indicating that addition of citrate failed to reduce iron limitation in this medium. It has been previously suggested that a hierarchy exists in the expression of iron acquisition systems in *P. aeruginosa*, placing those with the highest affinity for iron at the top (Hohnadal and Meyer 1988) and so it would be unlikely that the population would invest valuable resources upregulating the low affinity ferric-citrate transporter system when the high affinity pyoverdine system would be more productive in this medium. Furthermore, chelators such as citrate and ascorbate have been reported to increase the rate of iron autoxidation by 3 to 4-fold (Welch *et al.* 2003) and so may be actually reducing iron availability even further. *P. aeruginosa* is able to utilise a wide selection of compounds as a carbon source (Suh *et al.* 1999) which is conducive to its colonisation of diverse environments. Citrate has been shown to be a carbon source for *P. aeruginosa*, which has been suggested to be linked to the organism's ferric-citrate transporter (Cox 1980). Consequently, the failure of citrate to increase iron

availability in CDM_{10} complete may simply be because it is metabolised by the bacteria as a food source. Furthermore, cells grown in LB with citrate expressed a protein corresponding to the ferric citrate transporter but as LB is considered an iron replete medium, it is unlikely that this transporter is upregulated for iron acquisition, it is more likely involved acquisition of citrate as carbon source, which may be preferential to other sources available in the medium.

Ascorbate is also regarded as a low-affinity iron chelator but it cannot be used a carbon source by *P. aeruginosa* and although an ascorbate transporter has been reported in *E. coli* (Zhang *et al.* 2003) and the addition of ascorbate to aerobic cultures of *E. coli* increases oxygen uptake in minimal medium but reduces uptake in rich media (Richter *et al.* 1988), but as yet no such mechanisms have been reported in *P. aeruginosa*. Results revealed IROMP expression in cultures grown in CDM_{10} complete with 200 μM ascorbate, indicating that this medium provided no increase in iron availability. This correlates with the results achieved using citrate and confirms that these low affinity chelators cannot improve iron availability in CDM_{10} buffered with MOPSO and iron acquisition systems employing the high affinity siderophores, pyochelin and pyoverdine, are preferentially expressed in response to iron starvation.

4.11.5 The effect of MOPS and phosphate buffers on iron availability in CDM_{10} complete

An iron-replete phenotype has been demonstrated in *P. aeruginosa* culture grown in a defined minimal medium (Meyer *et al.* 1979) and when compared with CDM_{10} complete, the only significant difference in composition was the choice of buffer, with CDM_{10} using MOPSO, as opposed to phosphate buffer. A comparison of cells grown in MOPSO and phosphate buffered CDM_{10} revealed that an iron replete phenotype could be achieved when using phosphate buffer, indicating that iron availability was either reduced by MOPSO or increased by the high concentration of phosphate (100 mM).

Autoxidation can be defined as the direct combination of a substance with molecular oxygen at ordinary temperatures. Iron is particularly susceptible to this process in aerobic conditions whereby the equilibrium favours the oxidation of the ferrous iron into insoluble, ferric iron. Research has indicated that MOPS buffer does promote the autoxidation of iron in solution (Tadolini 1987a). The implications of this study were that MOPSO was responsible for the unavailability of iron in the media but alternatively the presence of high concentrations of phosphate (buffer) in the revised medium may actually improve the availability of iron in solution. For bacteria to acquire sufficient iron in the complex ligand environment of the growth medium, the iron must be buffered in such a way that it is relatively redox inactive (Welch *et al.* 2003). Although MOPS is reported to have a low affinity for iron, autoxidation occurs rapidly in this buffer and the addition of sodium phosphate to MOPS (Tadolini 1987b) significantly reduces the rate of autoxidation. Paradoxically, phosphate buffer has been reported to increase the rate of ferrous iron autoxidation in solution (Welch *et al.* 2003). Iron autoxidation has been reported in both MOPS and phosphate buffer occurs via two different pathways (Tadolini 1987a) and as iron solubility is dependent on its ligand environment, the components of CDM_{10} complete are perhaps more conducive to MOPS-dependent oxidation than that of phosphate explaining the differing iron availabilities between the two media.

4.11.6 The effect of medium buffer composition on pyoverdine production

Pyoverdine is the primary siderophore of *P. aeruginosa* and is exclusively expressed in response to iron-limited conditions (Stintzi *et al.* 1996) and there is an inverse relationship between iron content of a medium and the amount of pyoverdine produced (Meyer and Abdallah 1978). Quantification of pyoverdine levels by measurement of absorbance at 400 nm further consolidated the differences between CDM_{10} using MOPSO and phosphate buffer previously determined using SDS-PAGE. The highest pyoverdine levels were obtained in cultures grown in media containing no added iron, irrespective of buffer concentration. Elevated levels of pyoverdine were also detected in cultures grown in MOPSO-buffered CDM_{10} with 10 μM added iron, supporting the suggested iron-limited status of this medium. Whilst cultures grown in CDM_{10} buffered with phosphate showed minimal level of

absorbance indicating cells can obtain sufficient iron to support growth. These results revealed that measurement of pyoverdine levels not only supports data obtained using SDS-PAGE but also that this method could be used to determine the iron status of a culture accurately and rapidly.

4.11.7 Accuracy of SDS-PAGE

SDS-PAGE although an accurate method for determining protein size it does have its shortcomings; firstly it does lack sensitivity with large amounts of protein required for accurate bands densities and as nutrient limitation studies require cell growth to low densities very large culture volumes are required to produce sufficient protein for the assay. Secondly do to varying migration rates and gel compositions the actual molecular weight can be far from accurate as demonstrated by initially IROMPs in *P. aeruginosa* being identified as 70 to 80 kDa in size (Mizuno and Kageyama 1978) then subsequently identified as 80 to 90 kDa (Meyer *et al.* 1979). As the estimated protein size is the commonly used for the identification of proteins using SDS-PAGE the accuracy of the technique can become questionable. This can be rectified by extracting the outer membrane protein as normal and running it on a standard SDS gel, whereby bands can be visualized using standard methods. Subsequently the appropriate bands are blotted on a nitrocellulose membrane which is then probed with a specific antibody for the protein of interest and then visualised by Western blotting. This technique allows both the entire outer-membrane fraction to be visualised whilst more accurately confirming the identity of the protein of interest.

In conclusion, an iron-replete phenotype was achieved in *P. aeruginosa* cultures grown in CDM_{10} complete by replacing MOPSO with phosphate buffer, however it remains unclear by what mechanism iron was made unavailable in cultures buffered with MOPSO. This study established that specific nutrient-limitation in planktonic culture could be achieved whilst avoiding an iron-limited phenotype, by using CDM_{10} complete with phosphate buffer and 10 μM added iron. This would facilitate the next stage of study which would require both planktonic and biofilm cells grown under carbon, magnesium and iron-limited conditions.

5 THE USE OF DIRECT FLUORESCENCE MEASUREMENT TO MONITOR GENE EXPRESSION IN *P. aeruginosa* PAO1 *rpoS*::*gfp*mut3 FUSIONS

5.1 INTRODUCTION

Work in this study has indicated that the SMART assay is unable to provide the requisite sensitivity to investigate *rpoS* expression in *P. aeruginosa* at the chromosomal level (chapter 3). Although SMART is not prone to the contamination problems that can occur in PCR, the assay itself can be time consuming and the degree of stress the cells encounter in the processing steps may provide an inaccurate representation of gene expression. Therefore, a technique was required which would provide the requisite level of sensitivity but minimised the cellular stress.

The *gfp*mut3 mutant green fluorescent protein of *Aequorea victoria* (Andersen *et al.* 1998) provides a higher level of fluorescence compared to the wild-type protein (Tsien 1998). Consequently a *gfp*mut3 fusion expressed in a high copy number plasmid would hopefully generate sufficient signal to be detected using standard spectrofluorometry. This method has been used to monitor real-time gene expression using a 96-well spectrofluoremeter and results were comparable to those achieved using the *araBAD* operon fusions (Lu *et al.* 2002). Also monitoring of *gfp* fluorescence in conjunction with *lux* luminescence in a 96-well format has been shown to be comparable to conventional methods of monitoring gene expression (Lehtinen *et al.* 2003). Using a plasmid-borne *rpoS*::*gfp*mut3 fusion, cultures could be sampled through the growth cycle and OD and fluorescence measured directly, without requiring stressful processing steps. If the assay proved to be reproducible, investigation could be continued using chromosomal *rpoS*::*gfp*mut3 fusions, which would be more representative of *rpoS* expression at the cellular level

The growth of bacteria in sessile, surfaced-attached communities or biofilms, is a major cause of nosocomial infection (Costerton 2001) and cells growing in biofilms are reported to be 10-1000 times more resistant to antimicrobials than cells growing

planktonically (Mah and O'Toole 2001). Differences in both physiology and expression profiles between biofilm and planktonic growth suggest that a population's response to stress varies depending on its mode of growth. Although studies have addressed differences in gene expression between planktonic and biofilms cells (Prigent-Combaret *et al.* 1999), research has not been conducted using a chemically defined medium or even fully standardised conditions. Therefore, to investigate stress-induced *rpoS* expression, experiments would be conducted using a *P. aeruginosa* chemically defined medium, in conditions in which both planktonic and biofilm growth were as comparable as possible.

The stationary phase sigma factor RpoS has been identified as the main regulator of the stress response in *E. coli*, providing cross-protection for many environmental stresses including heat, oxidation, desiccation and changes in pH (Lange and Hengge-Aronis 1994a). RpoS in *E. coli* is regulated at a transcriptional, translational and proteolytic level (reviewed Hengge-Aronis 2000) and although RpoS levels in *P. aeruginosa* have been shown to increase on entry into stationary phase (Fujita *et al.* 1994), the complexities of its cellular regulation are less well-characterised than other gram-negative bacteria. The transcription of *rpoS* has been shown to be under the control of the Las and Rhl quorum sensing systems (Latifi *et al.* 1996), indicating that its expression is controlled, at least in part, by cell density. Other global regulators such as Vfr (West *et al.* 1994) and inorganic polyphosphate (Shiba *et al.* 1997) have also been implicated in its regulation. The effects of starvation of a wide selection of nutrients have been investigated in *E. coli* (Loewen 1984) and the effects on *rpoS* expression have been determined (Hengge-Aronis 2002), however, there has been a paucity of research addressing this subject in *P. aeruginosa*, with only the effects of carbon (Suh *et al.* 1999) and phosphate starvation (Jorgensen *et al.* 1999) being fully investigated. Therefore further investigation of how a major environmental stress such as nutrient limitation affects *rpoS* expression in *P. aeruginosa* was required.

The aims of this study were to:

- Determine if direct fluorescence measurement of plasmid and chromosomal *rpoS*::*gfp*mut3 fusions would provide an accurate representation of *rpoS* expression through the growth cycle

- Compare the effects of carbon, magnesium and iron limitation with cells grown in nutrient replete conditions

- Determine any differences in *rpoS* expression between nutrient-limited biofilm and planktonic culture

5.2 THE EXPRESSION OF *rpoS::gfp*mut3 IN NUTRIENT-REPLETE CULTURE

5.2.1 The expression of plasmid-borne *rpoS::gfp*mut3 fusion in nutrient-replete planktonic culture

The growth of SS225 (*P. aeruginosa* PAO1/pUCP20; control strain containing blank plasmid) and SS429 (*P. aeruginosa* PAO1/pSS429; *rpoS*::*gfp*mut3 plasmid fusion strain) in CDM_{10} complete was investigated. Fig. 5.1 A. reveals that both strains showed steady log-phase growth with cells entering stationary phase at a density of approximately 6 x 10^9 cells/ml, which resulted in growth cessation at 7.5 x 10^9 cells/ml. Fig. 5.1 B. illustrates that SS225 fluorescence steadily increased from inoculation, through log phase and peaked at 63 fu (fluorescence units)/million cells as the population entered stationary phase. After this point there was no further increase in fluorescence. A similar pattern was noted for SS429 (Fig 5.1 B.), with a steady increase in fluorescence from inoculation, through log phase and peaking at 303 fu/million cells as cells entered stationary phase. Although the inoculum was taken from a log-phase culture starter culture, results indicate the initial carry-over of fluorescence was high and remained so throughout log-phase growth.

5.2.2 The expression of chromosomal *rpoS::gfp*mut3 fusion in nutrient-replete planktonic culture

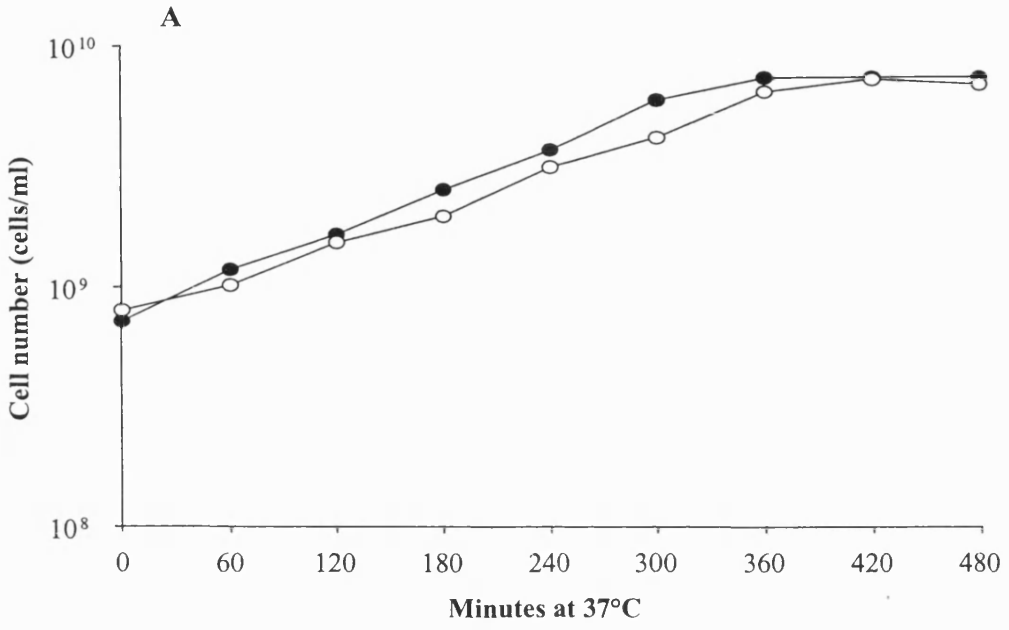
Examination of the growth cycle of *P. aeruginosa* PAO1 and SS336 (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3 chromosomal fusion) in CDM_{10} complete (Fig. 5.2 A.) revealed a gradual reduction in growth rate as cells entered stationary phase at a density of approximately 3 x 10^9 cells/ml resulting in growth cessation at 8 x 10^9 cells/ml. SS336 displayed no increase in fluorescence above background levels from inoculation until approximately two generations before stationary phase (Fig. 5.2 B.). From this point fluorescence steadily increased until growth cessation, reaching a maximum fluorescence of 102 fu/million cells compared to 59 fu/million cells noted in *P. aeruginosa* PAO1 cultures at the corresponding point of the growth cycle. This two-fold increase in fluorescence suggested that in nutrient-

replete conditions, the expression of *rpoS*::*gfp*mut3 was upregulated as cells entered to stationary phase.

5.2.3 The expression of chromosomal *rpoS::gfp*mut3 fusion in nutrient-replete biofilm culture

The growth of *P. aeruginosa* PAO1 and SS336 biofilms on CDM_{10} complete agar (Fig 5.3 A.) revealed a similar profile to that of planktonic culture, steady log-phase growth followed by a gradual reduction in growth rate with cells entering stationary phase at 2 x 10^9 cells/biofilm which resulted in growth cessation at 9 x 10^9 cells/biofilm. Fig 5.3 B. illustrates that the fluorescence of SS336 did not increase above background levels through log phase and early stationary phase growth. An increase in fluorescence was noted at the point of growth cessation reaching a maximum of 32 fu/million cells compared with 23 fu/million cells noted in *P. aeruginosa* PAO1 at the corresponding point of the growth cycle. This suggested a modest increase in *rpoS* expression as cells were approaching growth cessation in nutrient-replete conditions

Fig. 5.1 The expression of a *P. aeruginosa* PAO1 plasmid-borne *rpoS-gfp*mut3 fusion grown in CDM_{10} complete. **A**. Planktonic growth of SS225 (○), (*P. aeruginosa* PAO1/pUCP20; control strain containing blank plasmid) and SS429 (●), (*P. aeruginosa* PAO1/pSS429; *rpoS*::*gfp*mut3 plasmid fusion strain) in CDM_{10} complete. **B**. Fluorescence of *P. aeruginosa* SS225 (white bars) and SS429 (black bars). n=3 ±SEM.. All test values were significantly higher than control values, $P \leq 0.05$



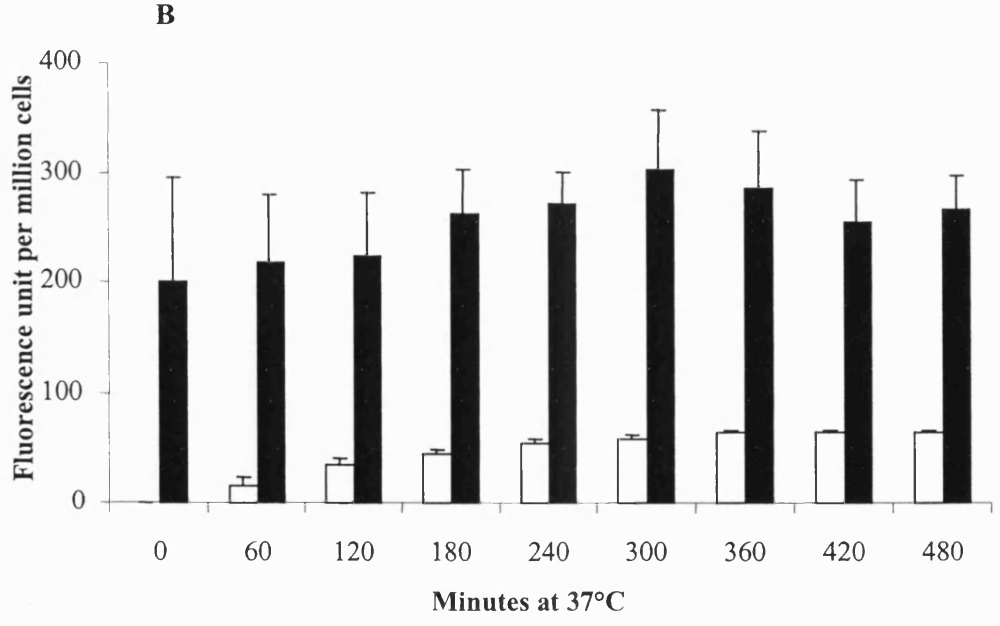
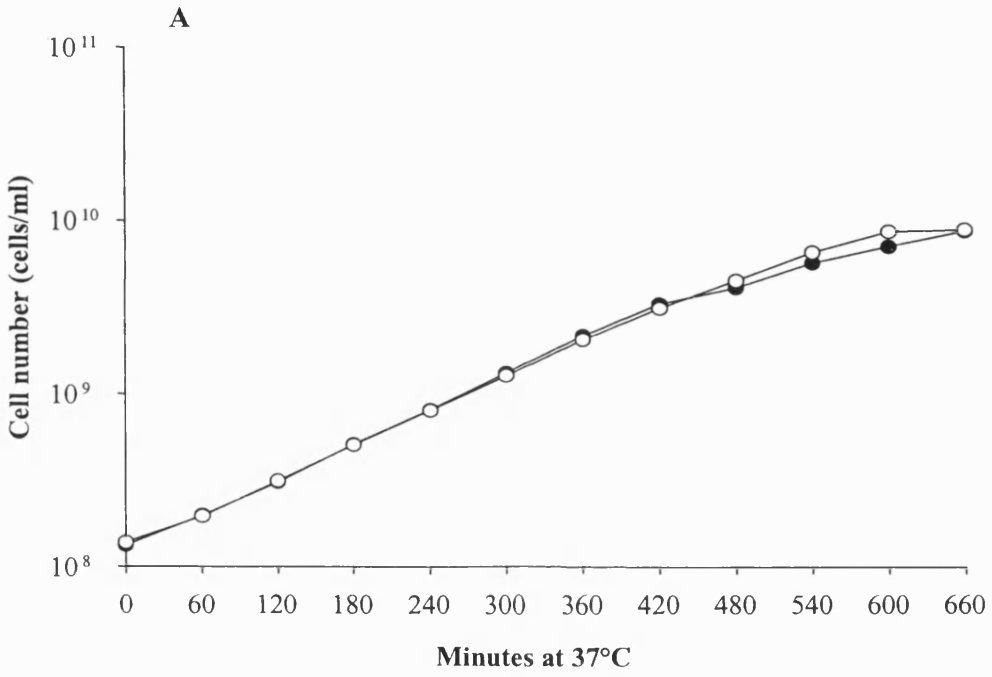


Fig. 5.2 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion grown in CDM_{10} complete. **A**. Planktonic growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) in CDM_{10} complete. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM. * P ≤0.05



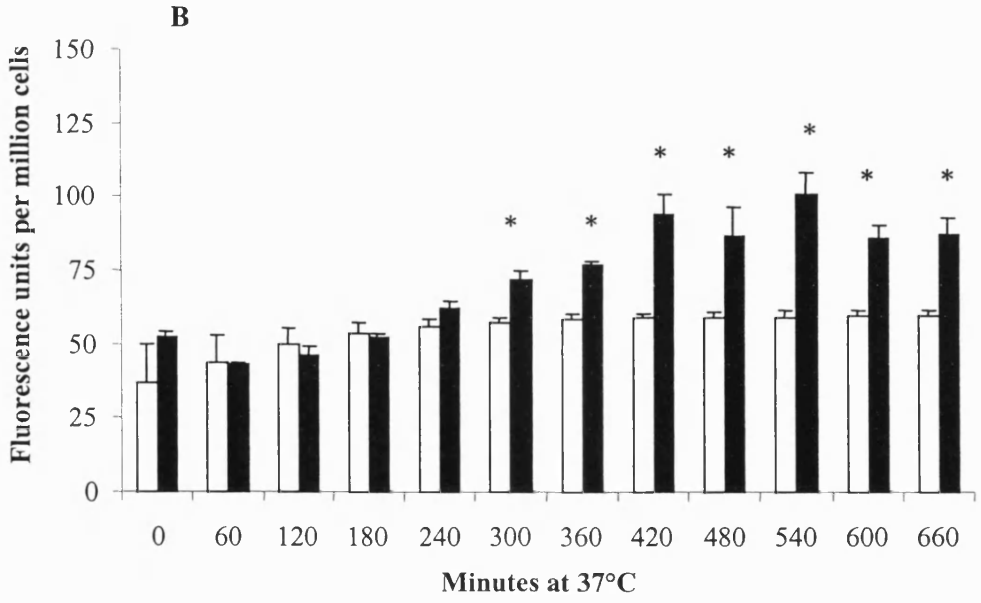
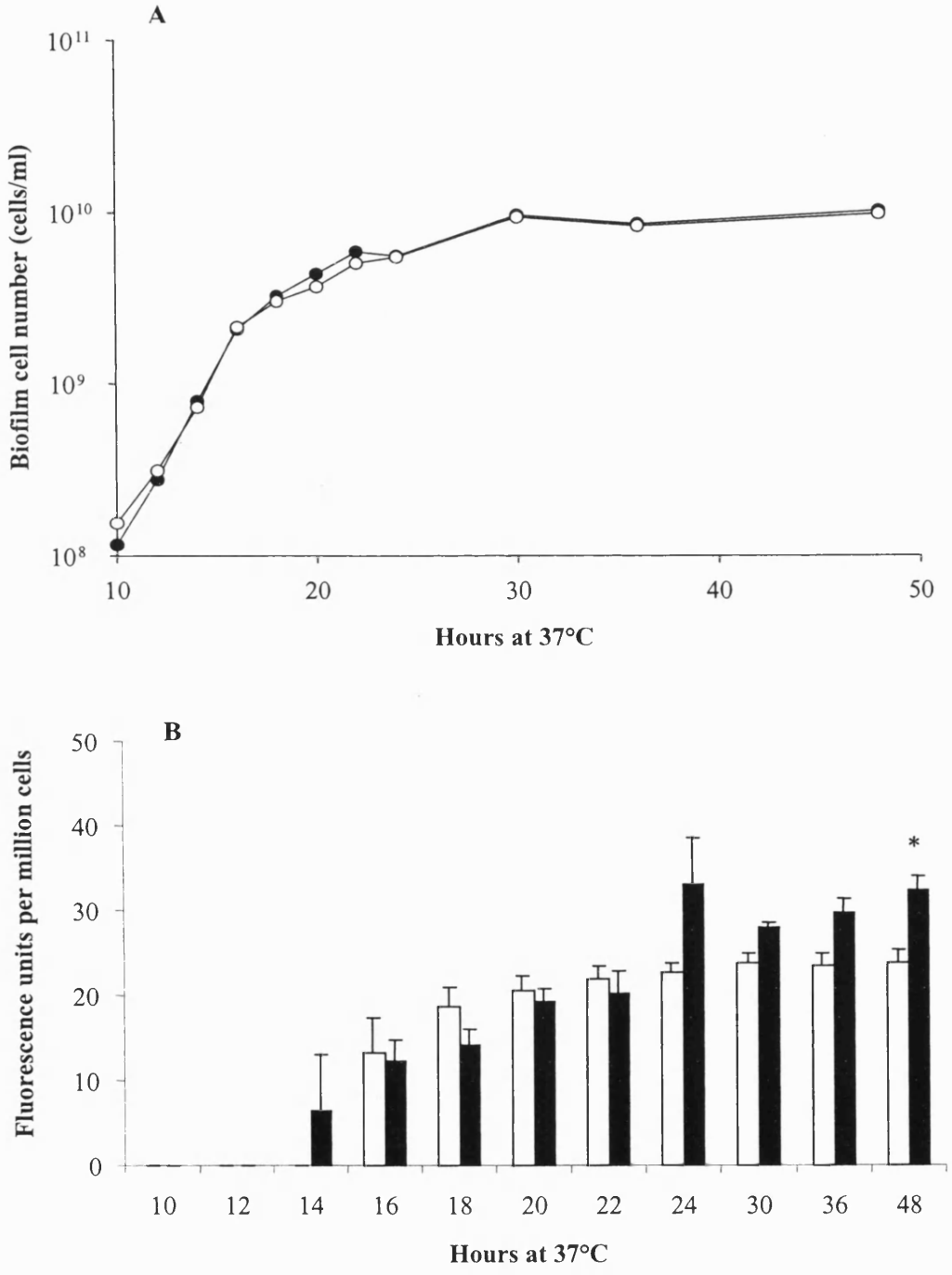


Fig. 5.3 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion grown as a biofilm on CDM_{10} complete agar. **A.** Biofilm growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) on CDM_{10} complete agar. **B.** Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM.



* P ≤0.05

5.3 THE EXPRESSION OF *rpoS::gfp*mut3 IN GLUCOSE-LIMITED CULTURE

5.3.1 Carbon limitation in planktonic culture

P. aeruginosa PAO1 cultures were grown in CDM containing 0, 1, 5, 10, 20, 40 and 100 mM glucose and stationary phase final densities were measured spectrophotometrically at 470 nm. Fig. 5.4 illustrates that glucose concentrations below 20 mM determined the density at which the cells entered stationary phase. At glucose concentrations exceeding 20 mM, cell growth was limited by some other factor.

5.3.2 Carbon limitation in biofilm culture

P. aeruginosa PAO1 biofilm cultures were grown on CDM agar containing 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 70 and 100 mM glucose and the stationary phase final density following dispersion was measured at 470 nm. Fig. 5.5 illustrates that glucose concentrations below 40 mM directly affected the density at which the cells entered stationary phase. At glucose concentrations exceeding 40 mM, cell growth was limited by some other factor. Results indicated that at concentrations below 7 mM, the linear relationship between cell density and glucose concentration was not proportional, perhaps due to contaminating levels of glucose in the agar.

5.3.3 The expression of plasmid *rpoS::gfp*mut3 fusion in glucose-limited planktonic culture

Examination of the growth cycle of *P. aeruginosa* SS225 and SS429 in CDM containing 2 mM glucose (Fig. 5.6 A.) revealed an abrupt cessation of growth and consequent entry stationary phase at 3 x 10^8 cells/ml. The prolonged reduction in growth rate observed in cells grown in CDM_{10} complete was not seen in glucose-limited conditions. The fluorescence of SS429 (Fig 5.6 B.) steadily increased from a high initial value at inoculation, peaking at 180 fu/million cells as the population entered stationary phase, compared to 51 fu/million cells noted in SS225. After this point there was no further increase in fluorescence.

5.3.4 The expression of chromosomal *rpoS::gfp*mut3 fusion in glucose-limited planktonic culture

Examination of the growth cycle of *P. aeruginosa* PAO1 and SS336 in CDM containing 2 mM glucose (Fig. 5.7 A.) revealed an abrupt cessation of growth and consequent entry stationary phase at a density of 1.7 x 10^9 cells/ml. Fig. 5.7 B. illustrates that in glucose-limited conditions the fluorescence of SS336 did not increase above background levels suggesting minimal *rpoS* expression in glucose-limited conditions.

5.3.5 The expression of chromosomal *rpoS*::*gfp*mut3 fusion in glucose-limited biofilm culture

The growth curves of *P. aeruginosa* PAO1 and SS336 biofilms grown on CDM agar containing 15 mM glucose (Fig 5.8 A.) reveal a gradual reduction in growth rate as cells enter stationary phase at 2 x 10^9 cells/biofilm, followed by growth cessation at 7 x 10^9 cells/biofilm. The fluorescence of SS336 (Fig 5.8 B.) did not rise above background levels for the duration of the growth curve.

Fig. 5.4 The effect of glucose concentration on the growth yield of *P. aeruginosa* PAO1 in planktonic culture. Final stationary-phase cell densities of *P. aeruginosa* PAO1 planktonic cultures incubated at 37°C in CDM containing various concentrations of glucose. Cell density was measured spectrophotometrically at 470 nm. n=3.

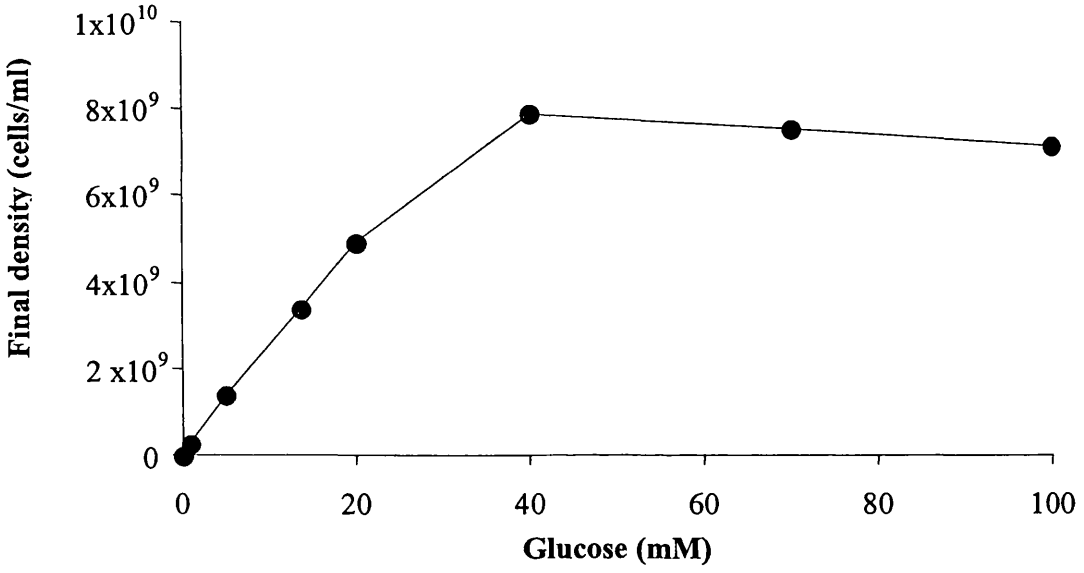


Fig. 5.5 The effect of glucose concentration on the growth yield of *P. aeruginosa* PAO1 in biofilm culture. Final stationary-phase cell densities of *P. aeruginosa* PAO1 biofilms incubated at 37°C for 48 h on CDM agar containing various concentrations of glucose. Cell density was measured spectrophotometrically at 470 nm. n=3.

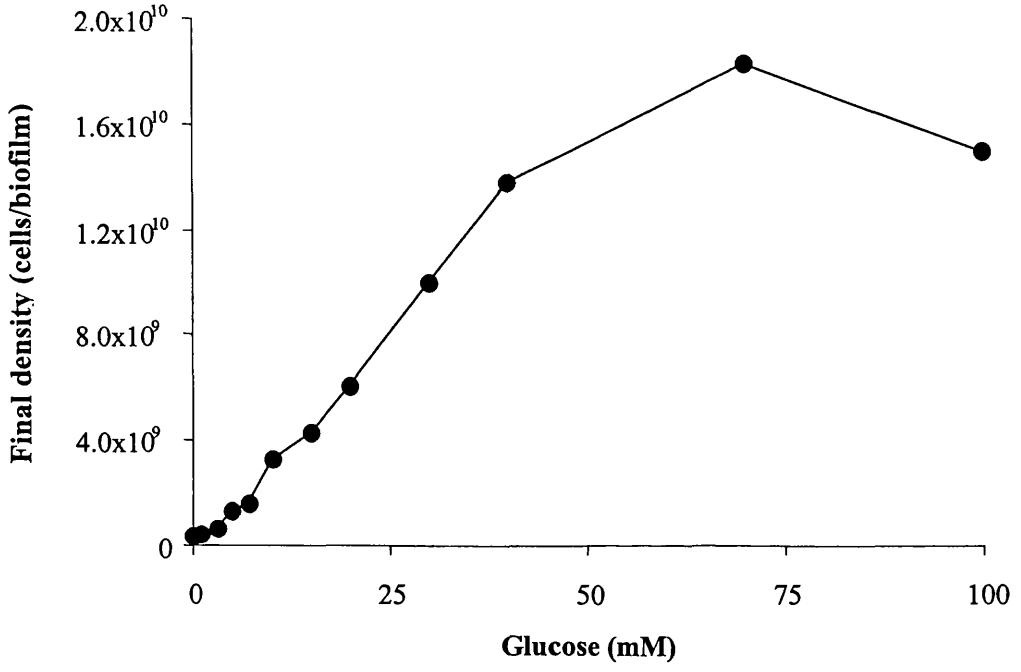
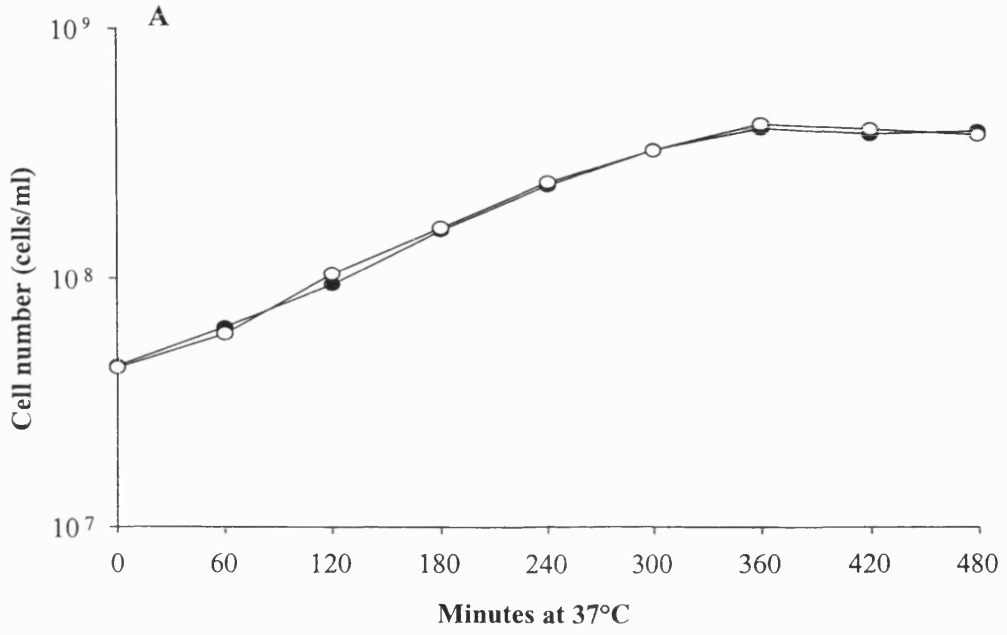


Fig. 5.6 The expression of a *P. aeruginosa* PAO1 plasmid-borne *rpoS-gfp*mut3 fusion grown in glucose-limited CDM. **A**. Planktonic growth of SS225 (○), (*P. aeruginosa* PAO1/pUCP20; control strain containing blank plasmid) and SS429 (●), (*P. aeruginosa* PAO1/pSS429; *rpoS*::*gfp*mut3 plasmid fusion strain) in CDM containing 2 mM glucose. **B**. Fluorescence of *P. aeruginosa* SS225 (white bars) and SS429 (black bars). n=3 ±SEM. All test values were significantly higher than control values, $P \leq 0.05$



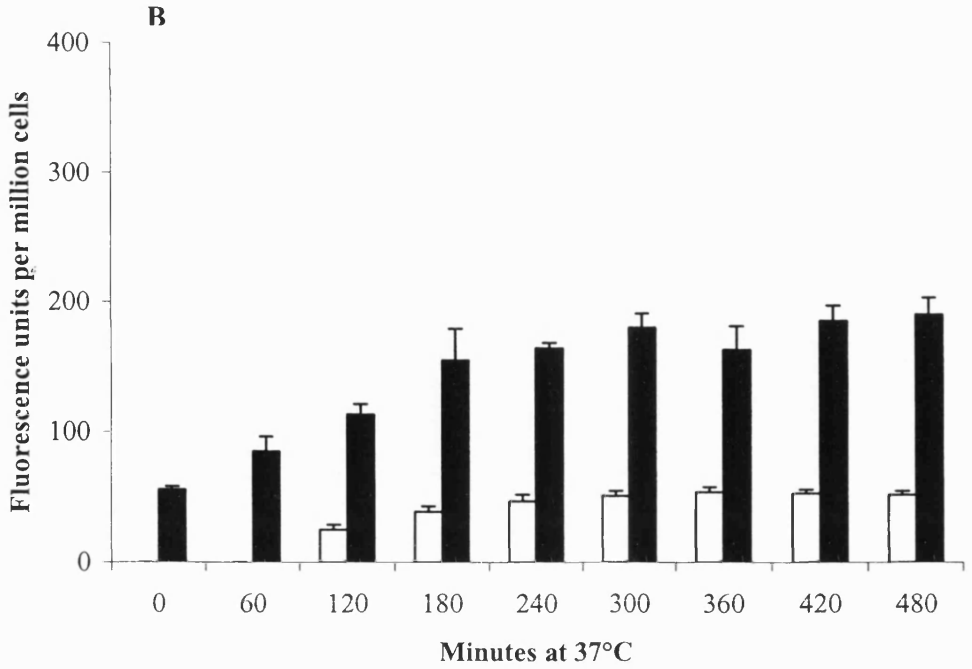
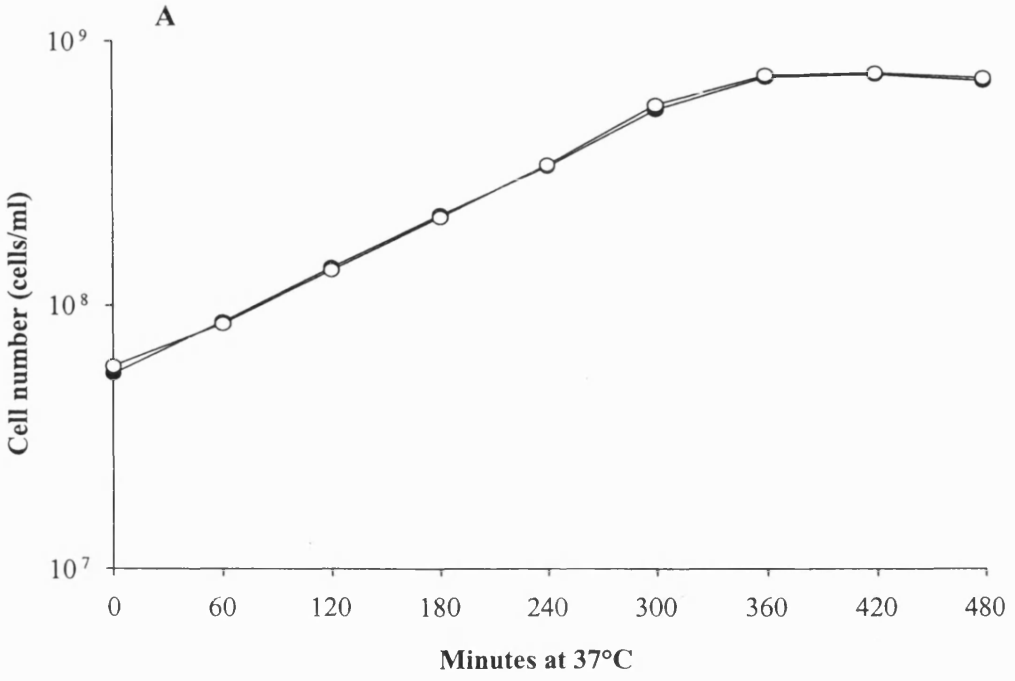


Figure 5.7 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion grown in glucose-limited CDM. **A**. Planktonic growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) in CDM containing 2 mM glucose. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM.



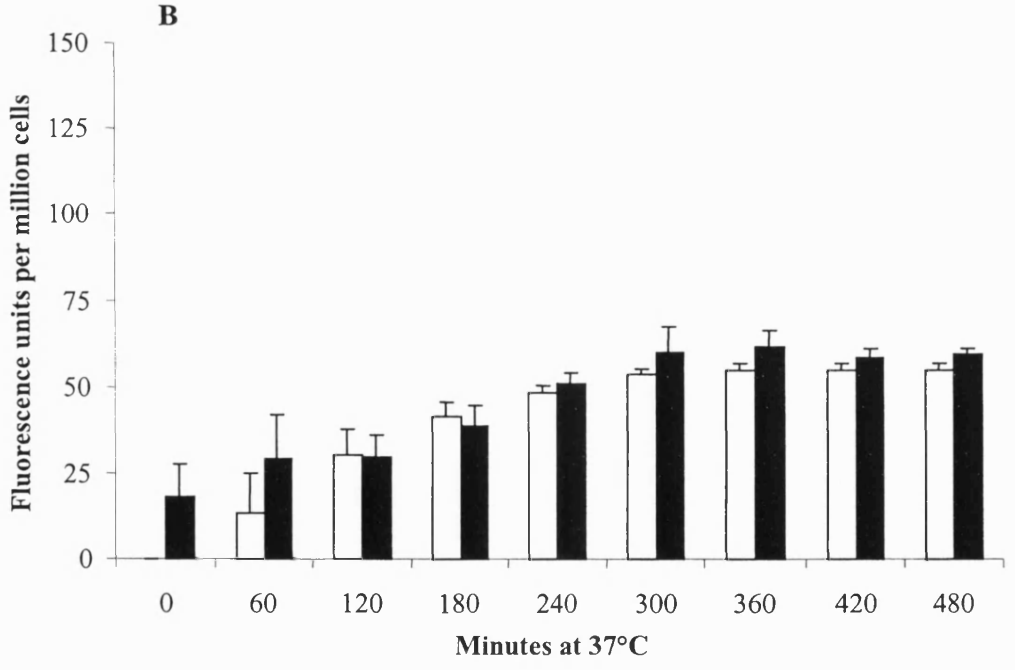
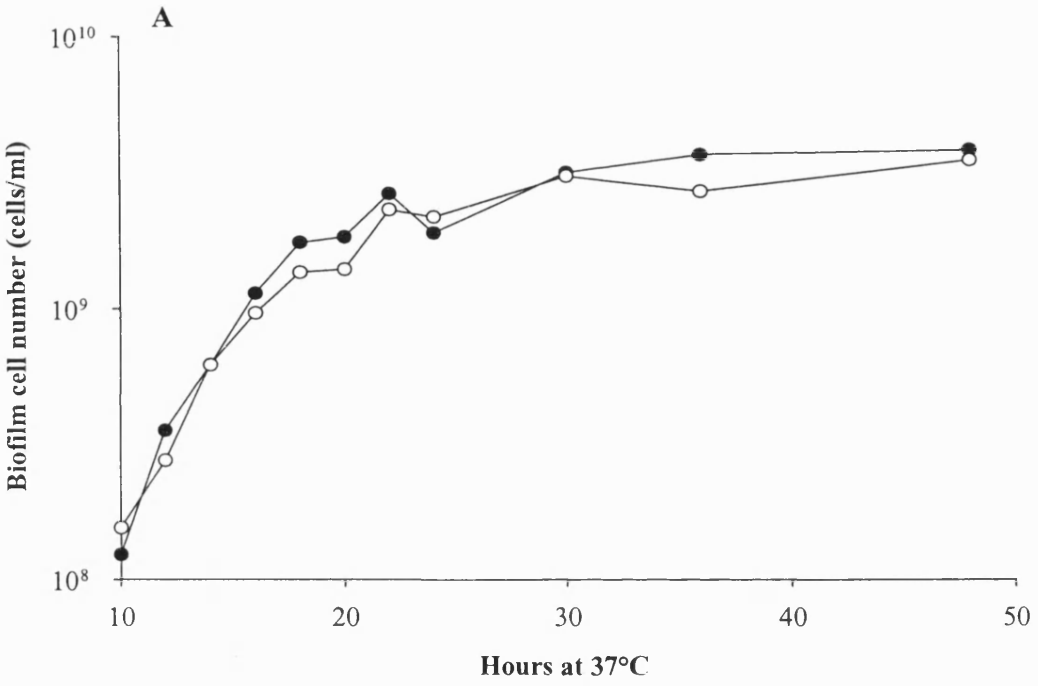
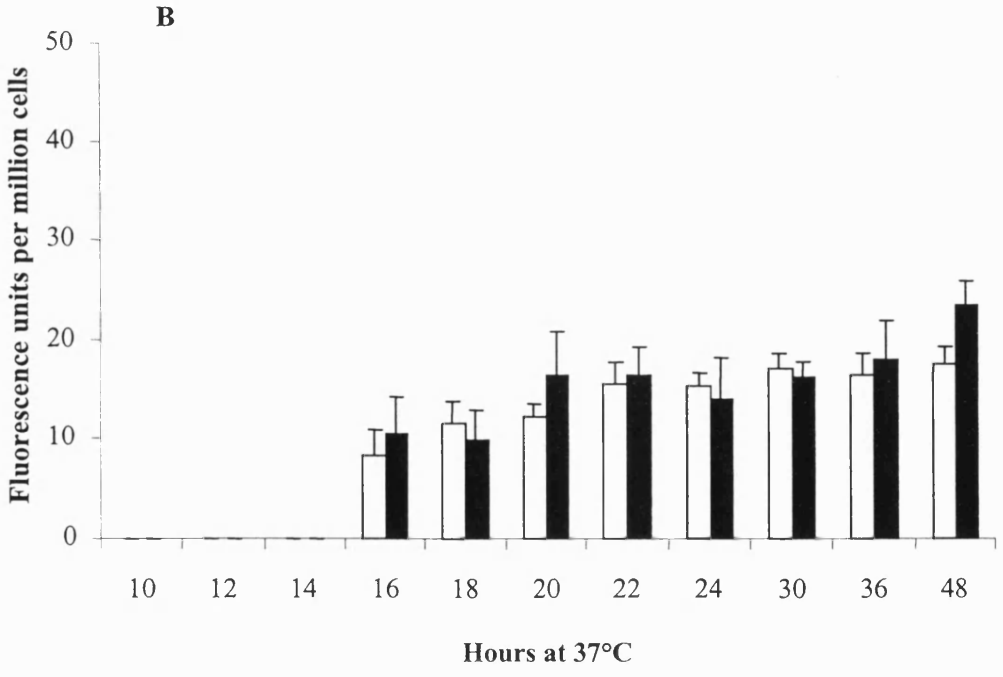


Figure 5.8 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion biofilms on glucose-limited CDM agar. **A**. Biofilm growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) on CDM agar containing 15 mM glucose. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4±SEM.





5.4 THE EXPRESSION OF *rpoS*::*gfp*mut3 IN MAGNESIUM-LIMITED CULTURE

5.4.1 Magnesium limitation in planktonic culture

P. aeruginosa PAO1 cultures were grown in CDM containing 0, 10, 30, 50, 100, 300, 500, 800 and 1000 μM magnesium chloride and stationary phase final densities were measured spectrophotometrically at 470 nm. Fig. 5.9 illustrates that magnesium concentrations below 60 μM directly determined the density at which the cells entered stationary phase. At magnesium concentrations exceeding 60 μM, cell growth was limited by some other factor.

5.4.2 Magnesium limitation in biofilm culture

P. aeruginosa PAO1 cultures were grown in CDM containing 0, 30, 50, 60, 70, 85, 100, 150, 400, 700 and 1000 μM magnesium and the stationary phase final density, following dispersion, was measured at 470 nm. Fig. 5.10 illustrates that magnesium concentrations below 100 μM directly affected the density at which the cells entered stationary phase. At magnesium concentrations exceeding 100 μM, cell growth was limited by some other factor. At concentrations below 30 μM, the relationship between magnesium concentration and final density was not linear, possibly due to chelation by the agar.

5.4.3 The expression of plasmid *rpoS*::*gfp*mut3 fusion in magnesium-limited planktonic culture

Examination of the growth cycle of *P. aeruginosa* SS225 and SS429 in CDM containing 3 μM magnesium chloride (Fig. 5.11 A.) revealed a gradual reduction in growth rate as cells enter stationary phase at cell density of approximately 4×10^8 cells/ml, which resulted in growth cessation at a density of 7×10^8 cells/ml. Fig 5.11 B. illustrates that in magnesium-limited conditions, the fluorescence of SS429 steadily increased from a high value at inoculation, through log-phase growth and continued to increase as cells entered stationary phase, reaching a maximum of 327 fu/million cells. This was much higher than 65 fu/million cells noted for *P. aeruginosa* PAO1 at the corresponding point of the growth cycle.

5.4.4 The expression of chromosomal *rpoS*::*gfp*mut3 fusion in magnesium-limited planktonic culture

Examination of the growth cycle of *P. aeruginosa* PAO1 and SS336 in CDM containing 3 μM magnesium chloride (Fig. 5.12 A.) revealed a gradual reduction in growth rate as cells enter stationary phase at cell density of approximately 6 x 10^8 cells/ml, resulting in growth cessation at a density of 1 x 10^9 cells/ml. Fig 5.12 B. illustrates that in magnesium-limited conditions, the fluorescence of SS336 did not increase above background levels until cells were in stationary phase whereupon a steady increase in fluorescence was noted. The population reached a maximum fluorescence of 68 fu/million cells compared to 44 fu/million cells noted in *P. aeruginosa* PAO1 at the corresponding point of the growth cycle.

5.4.5 The expression of chromosomal *rpoS*::*gfp*mut3 fusion in magnesium-limited biofilm culture

The growth of *P. aeruginosa* PAO1 and SS336 biofilms on CDM containing 15 μM magnesium chloride (Fig 5.13 A.) revealed a gradual reduction in growth rate as cells enter stationary phase at 8 x 10^8 cells/biofilm followed by growth cessation at 1.3 x 10^9 cells/biofilm. Fig 5.13 B. illustrates that the fluorescence of SS336 cells did not increase above background levels for the duration of the growth curve.

Fig. 5.9 The effect of magnesium concentration on the growth yield of *P. aeruginosa* PAO1 in planktonic culture. Final stationary-phase cell densities of *P. aeruginosa* PAO1 cultures incubated at 37°C in CDM containing various concentrations of magnesium chloride. Cell density was measured spectrophotometrically at 470 nm. n=3.

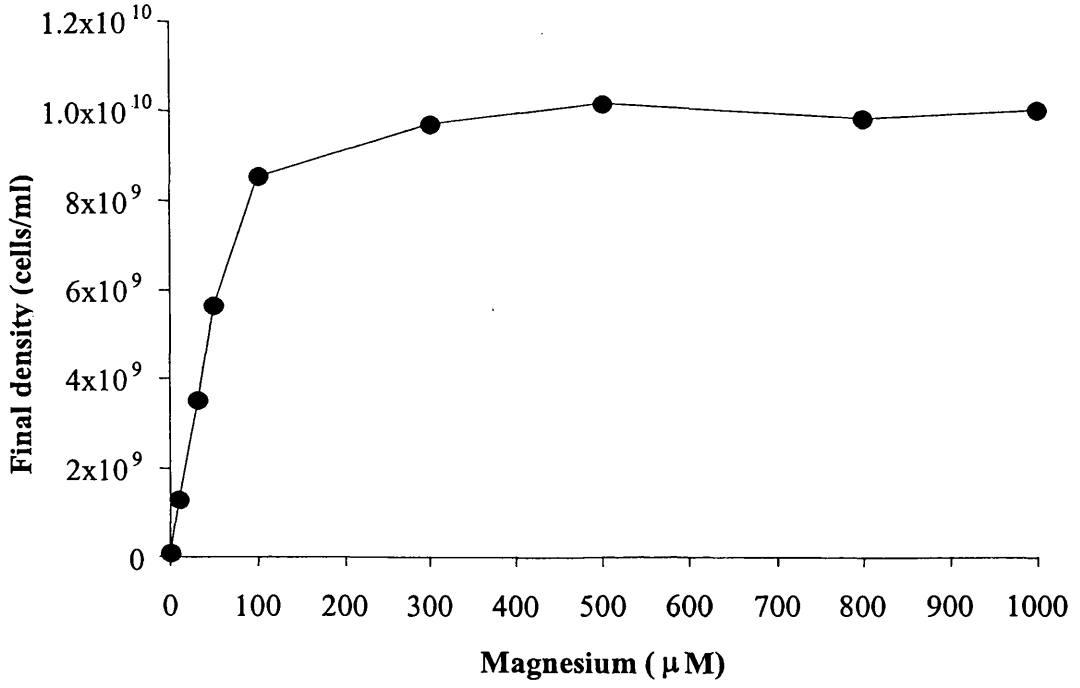


Fig. 5.10 The effect of magnesium concentration on the growth yield of *P. aeruginosa* PAO1 in biofilm culture. Final stationary-phase cell densities of *P. aeruginosa* PAO1 biofilm cultures incubated at 37°C for 48 h on CDM agar containing various concentrations of magnesium chloride. Cell density was measured spectrophotometrically at 470 nm. n=3.

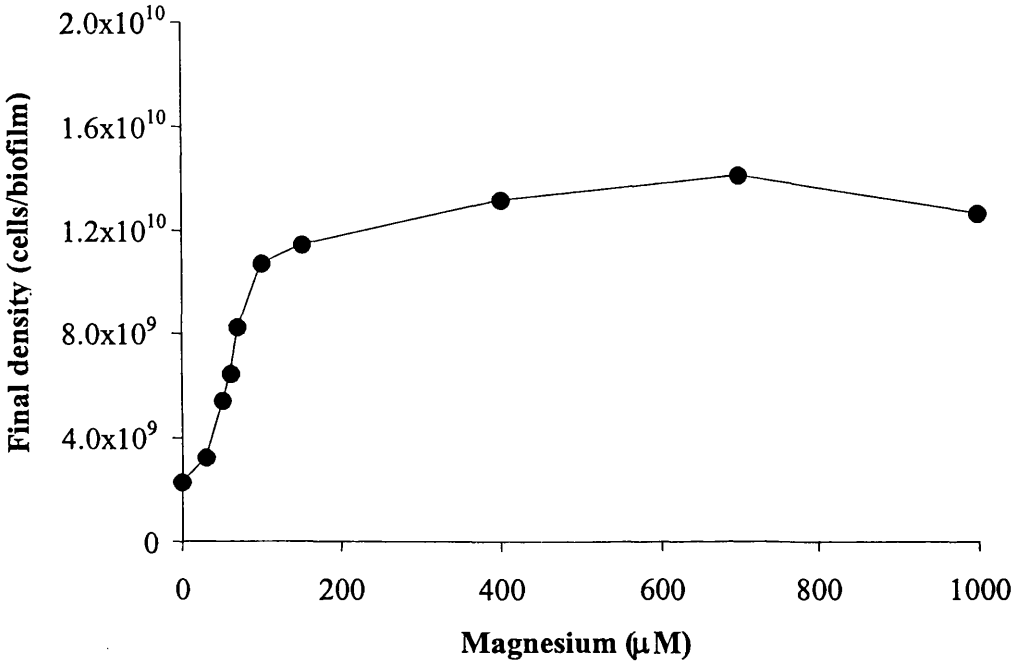
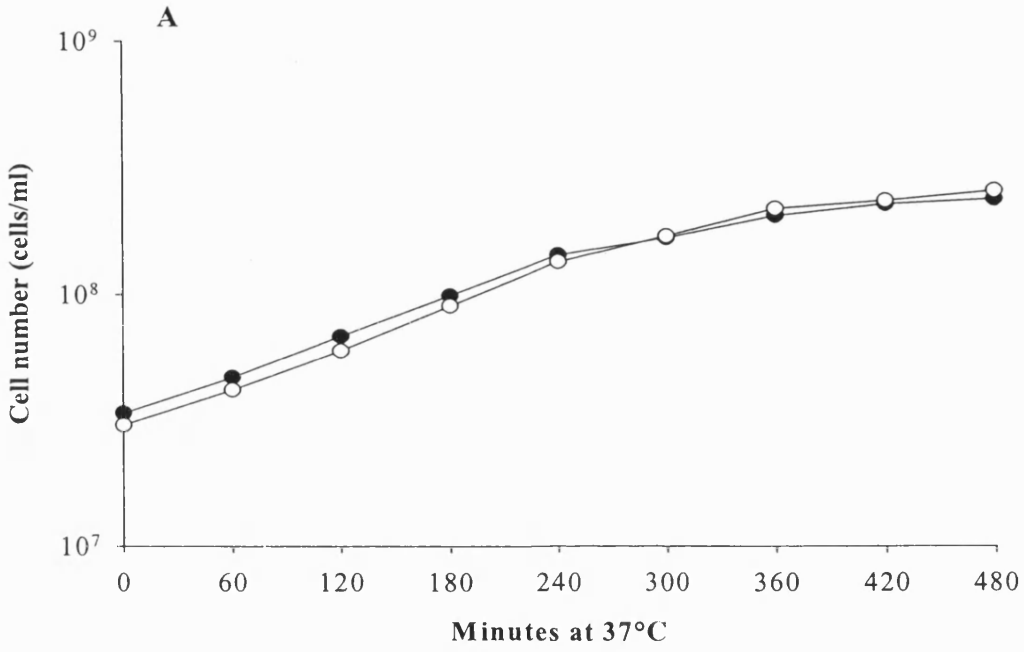


Fig. 5.11 The expression of a *P. aeruginosa* PAO1 plasmid-borne *rpoS-gfp*mut3 fusion grown in magnesium-limited CDM. **A**. Planktonic growth of SS225 (○), (*P. aeruginosa* PAO1/pUCP20; control strain containing blank plasmid) and SS429 (●), (*P. aeruginosa* PAO1/pSS429; *rpoS*::*gfp*mut3 plasmid fusion strain) in CDM containing 3 μM magnesium chloride. **B**. Fluorescence of *P. aeruginosa* SS225 (white bars) and SS429 (black bars). n=3 ±SEM. All test values were significantly higher than control values, $P \leq 0.05$.



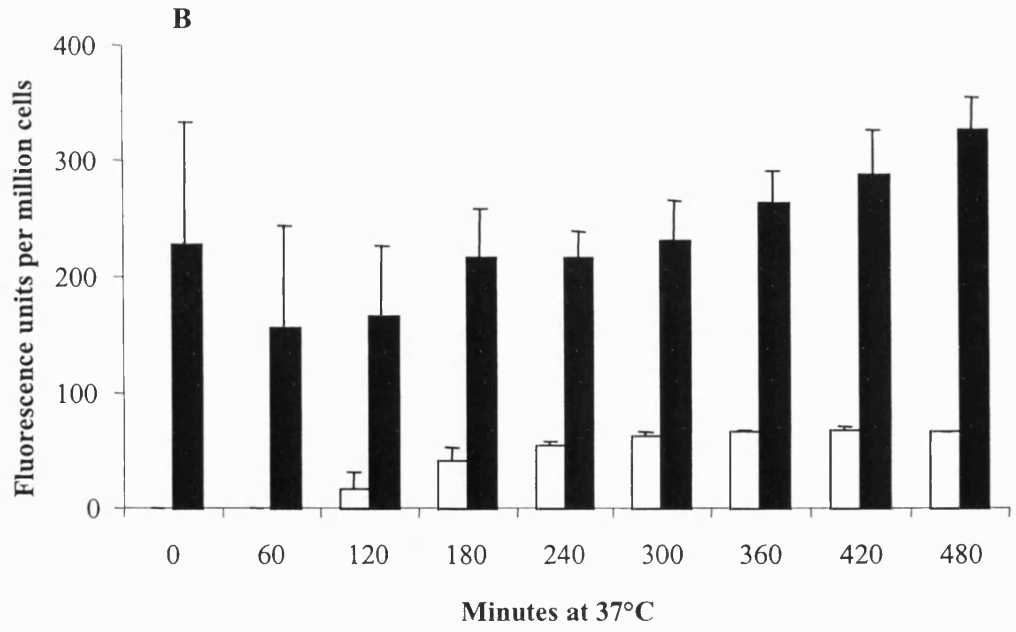
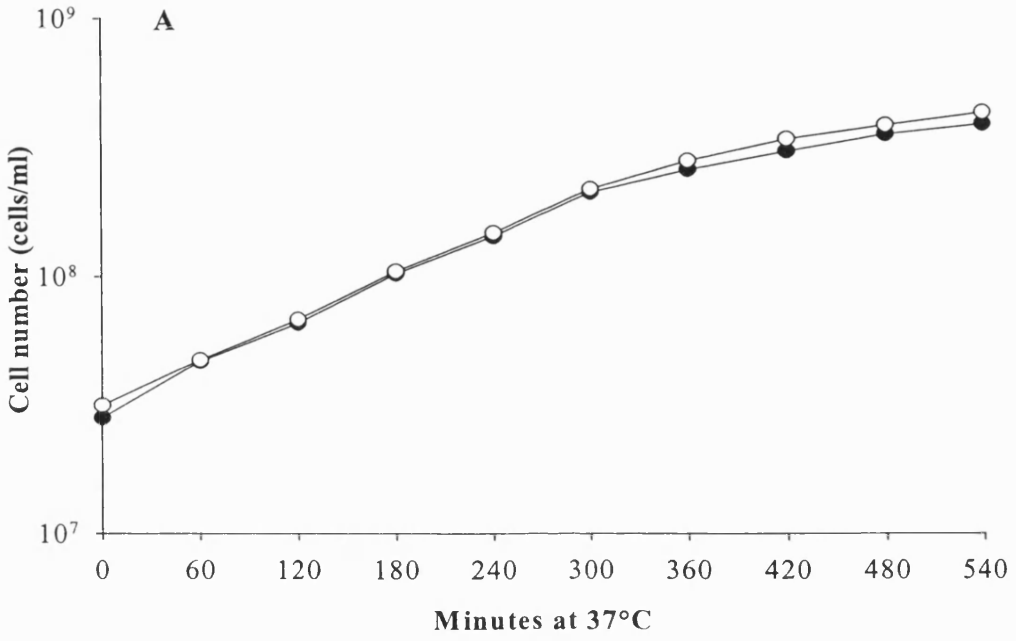
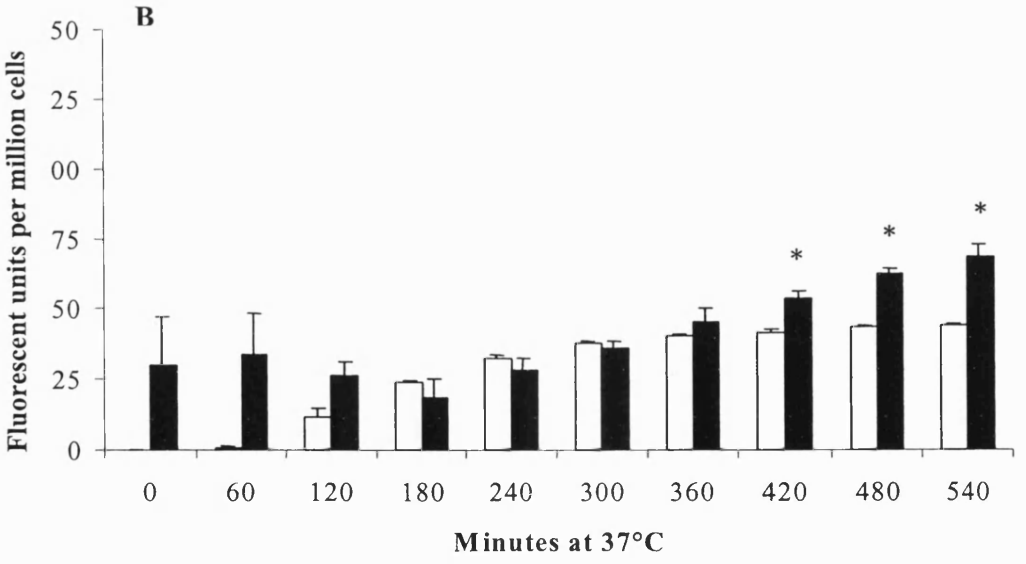


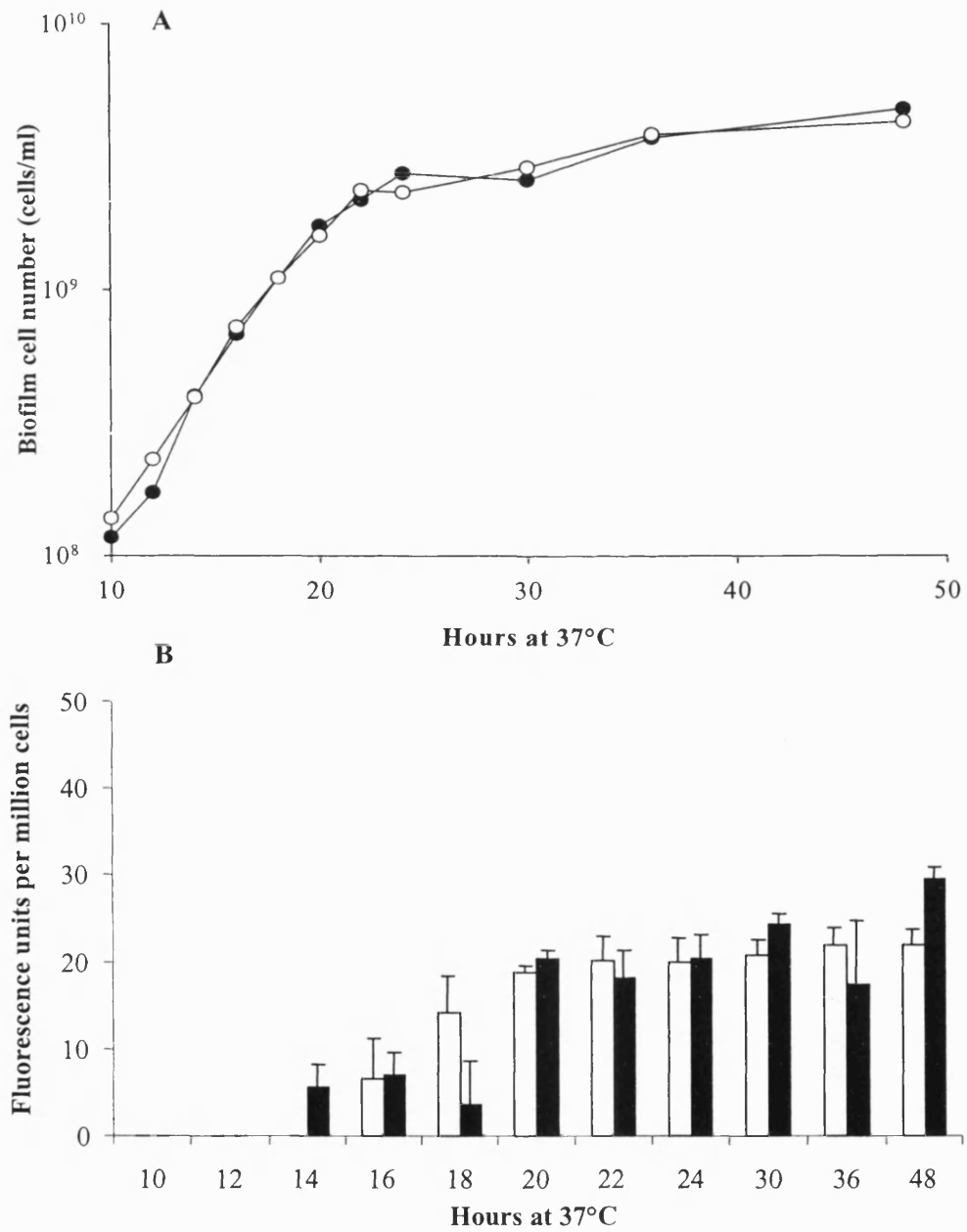
Fig 5.12 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion grown in magnesium-limited CDM. **A**. Planktonic growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) in CDM containing 3 μM magnesium chloride. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM





* P ≤0.05

Figure 5.13 The expression of a chromosomal *rpoS-gfp*mut3 fusion in *P. aeruginosa* PAO1 biofilms grown on magnesium-limited CDM agar A. Biofilm growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) on CDM agar containing 30 μM magnesium chloride. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM.



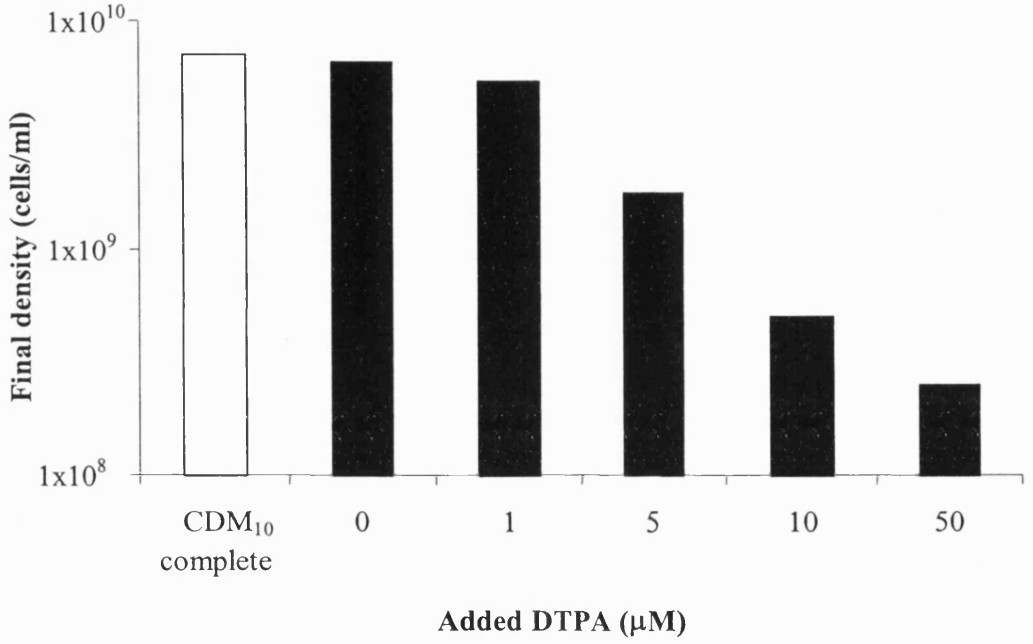
5.5 THE EXPRESSION OF *rpoS* IN IRON-LIMITED CULTURE

5.5.1 The effect of DTPA on the growth yield of *P. aeruginosa* PAO1

The removal of contaminating iron from media and glassware was problematic and even after steps such as acid washing and careful handling, cultures grown in CDM with no added iron were entering stationary phase at almost the same density as cells grown in CDM_{10} complete. Furthermore, even when iron limitation was achieved, the density at which cells did enter stationary phase was highly variable. Consequently, the chelator diethylenetriamine pentaacetic acid (DTPA) was selected to reduce iron availability in the medium and allow isolation of iron-limited cells.

Cultures were grown overnight at 37°C in CDM containing no added iron and a range of DTPA concentrations; CDM_{10} complete was used as a positive control. Results shown in Fig. 5.14 confirm that cultures with no added iron reached final densities comparable to cultures grown in CDM_{10} complete with 10 μM added iron In planktonic culture growth yield decreased proportionally to increasing concentrations of DTPA. Addition of 5 μM DTPA reduced the growth yield to a density of 1.5×10^9 cells/ml, which was considered suitable for nutrient limitation experiments. *P. aeruginosa* PAO1 biofilms grown on CDM agar with no added iron routinely reached densities lower that those obtained in nutrient-replete conditions. This suggested that the cells were iron limiting and consequently no DTPA was required

Fig. 5.14 The effect of DTPA of the growth yield of *P. aeruginosa* PAO1 cultures grown in CDM with no added iron. Final stationary-phase cell densities of *P. aeruginosa* PAO1 cultures incubated at 37°C in CDM containing various concentrations of DTPA; cell density was measured spectrophotometrically at 470 nm (black bars). CDM_{10} complete containing 10 μM iron was used as a control (white bar).



5.5.2 The expression of plasmid *rpoS*::*gfp*mut3 fusion in iron-limited planktonic culture

Examination of the growth cycle of *P. aeruginosa* SS225 and SS429 in iron-limited medium (Fig. 5.15 A.) revealed a gradual reduction in growth rate as cells enter stationary phase at a density of approximately 2.5 x 10^9 cells/ml. Fig 5.15 B. illustrates that in iron-limited conditions, the fluorescence of SS429 gradually increased from a high initial value at the point of inoculum, through log-phase and continued increasing through stationary phase. The fluorescence of SS429 reached a maximum of 234 fu/million cells compared to 75 fu/million cells for *P. aeruginosa* PAO1.

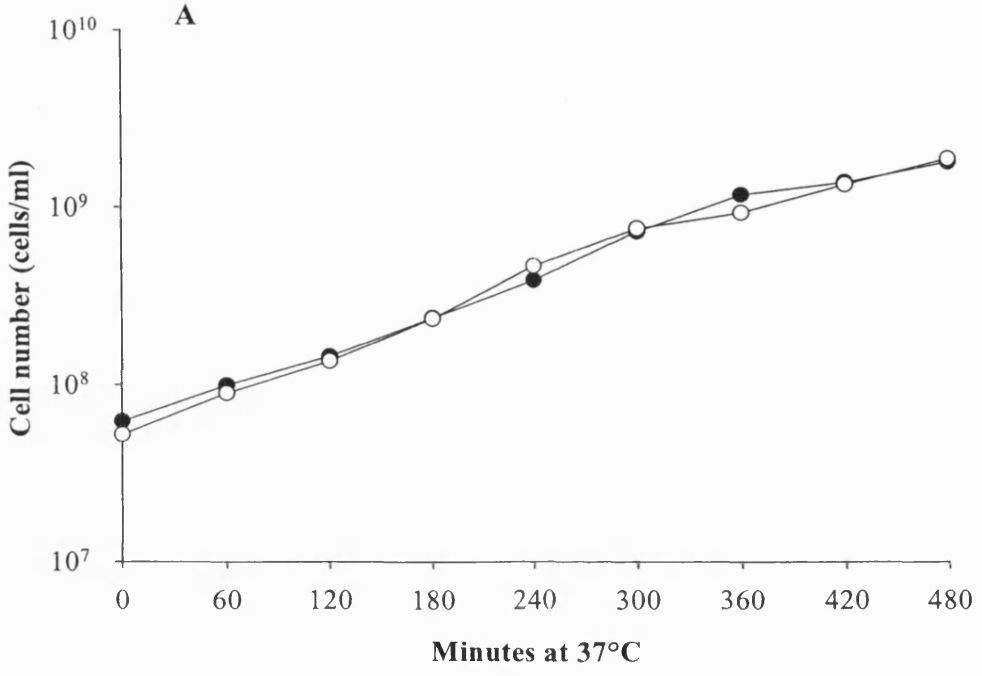
5.5.3 The expression of chromosomal *rpoS*::*gfp*mut3 fusion in iron-limited planktonic culture

Examination of the growth cycle of *P. aeruginosa* PAO1 and SS336 in iron-limited medium (Fig. 5.16 A.) revealed a gradual reduction in growth rate as cells enter stationary phase at cell density of approximately 3 x 10^9 cells/ml. Fig 5.16 B. indicates that even though the fluorescence of SS336 began increasing approximately three generations before stationary phase, the elevated fluorescence of *P. aeruginosa* PAO1 ensured that the SS336 cultures did not increase above background levels until the cells were established in stationary phase. At which point, the maximum fluorescence of 84 fu/million cells larger than 64 fu/million cells noted in *P. aeruginosa* PAO1.

5.5.4 The expression of chromosomal *rpoS*::*gfp*mut3 fusion in iron-limited biofilm culture

The growth of *P. aeruginosa* PAO1 and SS336 biofilms under iron-limited conditions (Fig 5.17 A.) reveals a gradual reduction in growth rate as cells enter stationary phase at a density of 3 x 10^9 cells/biofilm followed by growth cessation at a density of 9 x 10^9 cells/biofilm. Fig 5.17 B. illustrates that the fluorescence of SS336 cells did not increase above background levels for the duration of the growth cycle.

Figure 5.15 The expression of a *P. aeruginosa* PAO1 plasmid *rpoS::gfp*mut3 fusion grown in iron-limited CDM. **A**. Planktonic growth of SS225 (○), (*P. aeruginosa* PAO1/pUCP20; control strain containing blank plasmid) and SS429 (●), (*P. aeruginosa* PAO1/pSS429; *rpoS*::*gfp*mut3 plasmid fusion strain) in CDM containing 5 μM DTPA and no added iron. **B**. Fluorescence of *P. aeruginosa* SS225 (white bars) and SS429 (black bars). n=3 ±SEM. All test values were significantly higher than control values, $P \leq 0.05$



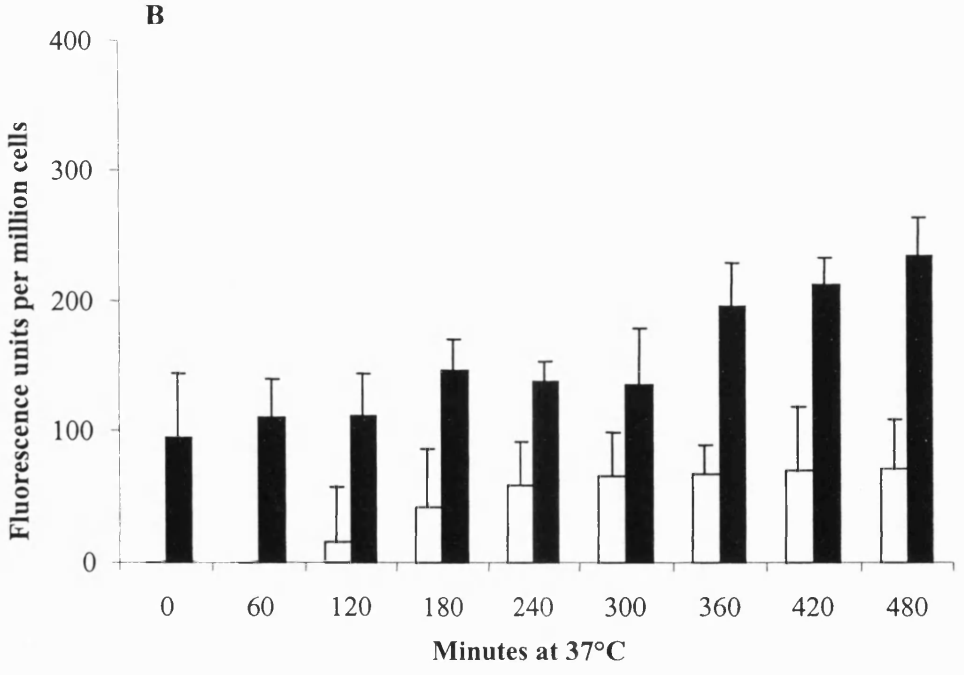
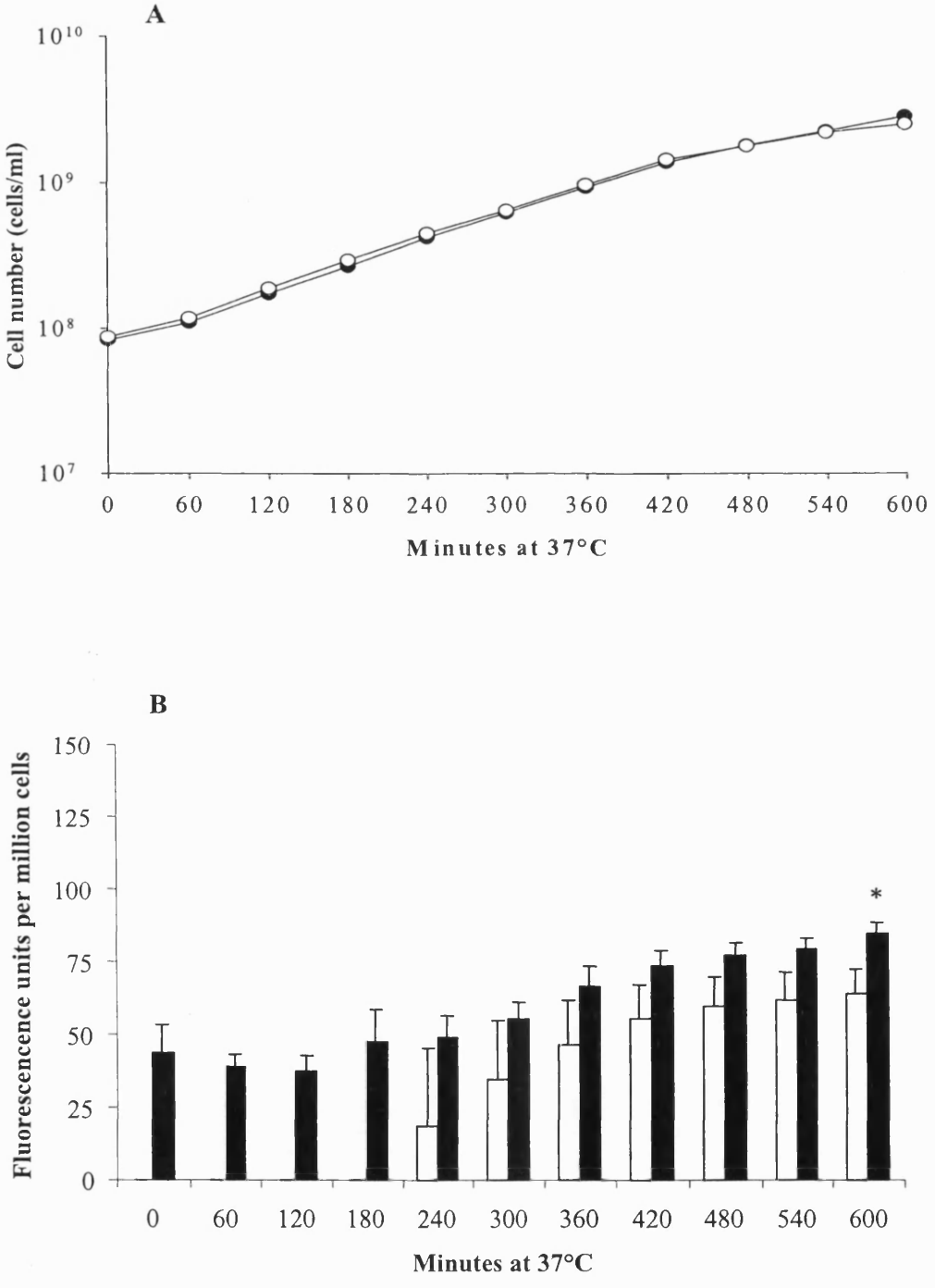
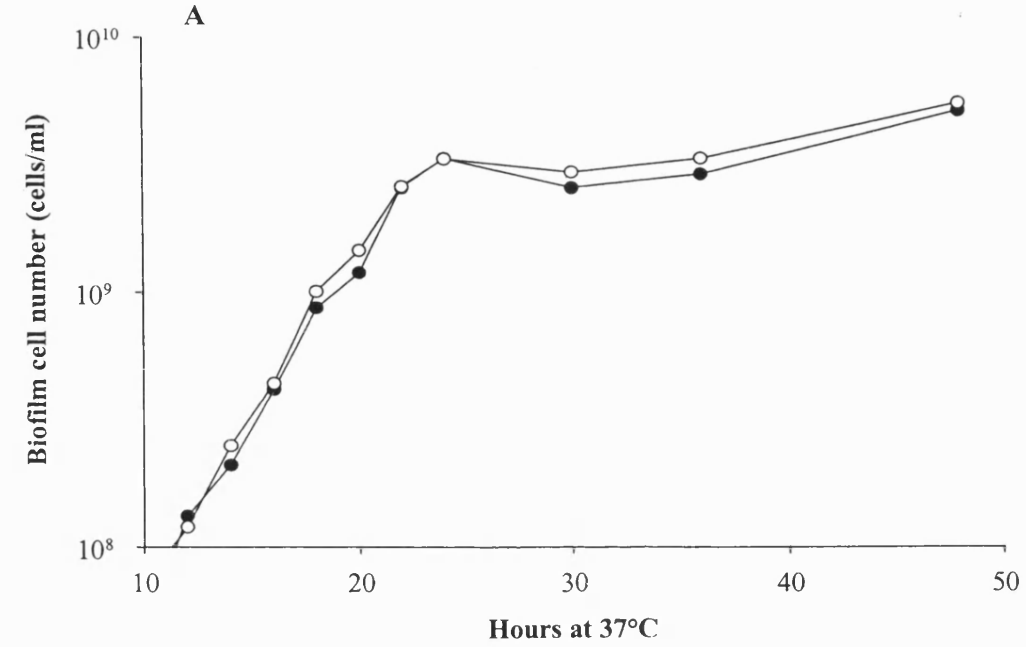


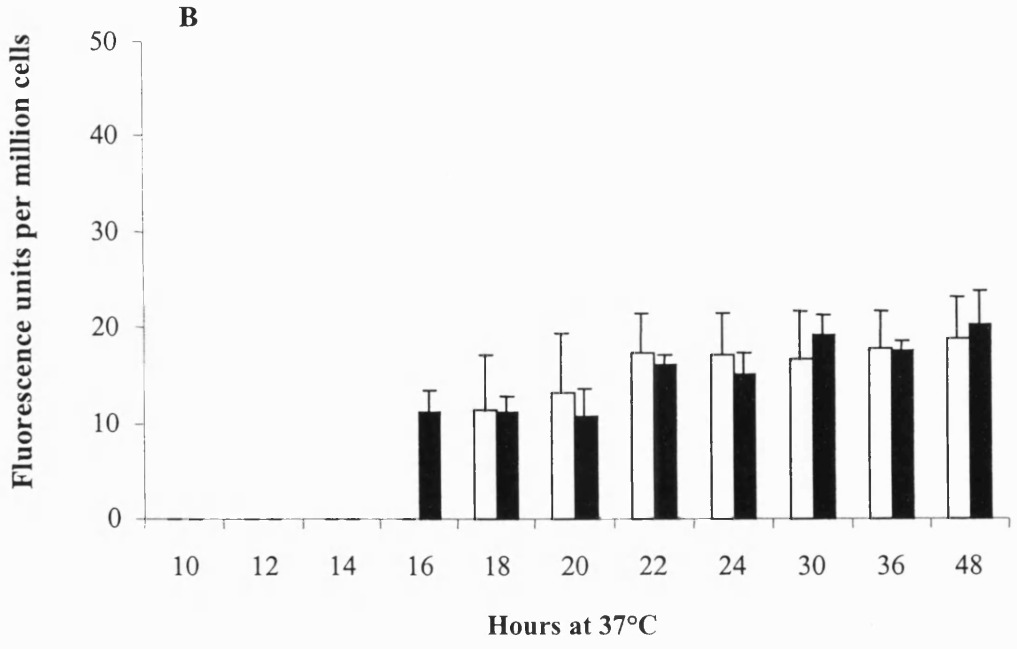
Figure 5.16 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion grown in iron-limited CDM. **A**. Planktonic growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) in CDM containing 5 μM DTPA and no added iron. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM.



* P ≤0.05

Figure 5.17 The expression of a *P. aeruginosa* PAO1 chromosomal *rpoS-gfp*mut3 fusion biofilms grown on iron-limited CDM agar. **A**. Biofilm growth of *P. aeruginosa* PAO1 (○), (wild-type strain) and SS336 (●), (*P. aeruginosa* PAO1 *rpoS*::*gfp*mut3; chromosomal fusion strain) on CDM agar with no added iron. **B**. Fluorescence of *P. aeruginosa* PAO1 (white bars) and SS336 (black bars). n=4 ±SEM.





5.6 DISCUSSION

The main aim of the project was to determine the suitability of using direct measurement of florescence of *gfp* fusions in "real time" without requiring any intermediate processing, thereby minimising stresses that could potentially affect the expression of *rpoS*. The following results were obtained in this study:

- The fluorescence of the plasmid-borne *rpoS*::*gfp*mut3 fusion increased proportionally with cell density from inoculation until stationary phase in nutrient-replete and glucose, magnesium and iron-limited conditions
- The fluorescence of the chromosomal *rpoS*::*gfp*mut3 fusion increased as cells entered stationary in nutrient-replete and magnesium-limited conditions whilst minimal increases were seen in both iron and glucose-limited conditions.
- Minimal increases in the fluorescence of the plasmid-borne *rpoS*::*gfp*mut3 fusion were noted in *P. aeruginosa* biofilms in glucose, magnesium and iron-limited conditions.

5.6.1 Intrinsic fluorescence of *P. aeruginosa*

A potential drawback to using *gfp* fusions to monitor gene expression in *P. aeruginosa*, as opposed to luciferase and β-galactosidase, is that *P. aeruginosa* produces fluorescent pigments such as pyoverdine and pyocyanin, in response to certain stimuli such as the approach of stationary phase and iron limitation (Meyer *et al.* 1979). The excitation and emission spectra of pyoverdine and wild-type GFP overlap (398 nm/470 nm and 395 nm/510 nm respectively; (Meyer and Abdallah 1978), (Cormack *et al.* 1996)) and although the mutations in the chromophore of *gfp*mut3 shifts excitation wavelength to 488 nm (Cormack *et al.* 1996), the fluorescence of the protein it would still be detected at wavelengths that would overlap with the intrinsic fluorescence of *P. aeruginosa*.

This study showed that the intrinsic fluorescence of *P. aeruginosa* PAO1 increases proportionally with cell density in planktonic culture. The fluorescence of the wild-type strain was monitored through the growth cycle so that the intrinsic

fluorescence of *P. aeruginosa* could be calculated at any cell density. This value was then subtracted from the fluorescence of the fusion strain, which resulted in fluorescence values solely attributable to the expression of *gfp*. The influence of intrinsic fluorescence is less pronounced when investigating high copy number fusions, which fluoresce at levels several orders of magnitude higher than background. However, the fluorescence of chromosomal fusions is detected at the lower end of the sensitivity range, at levels close to pigments produced by *P. aeruginosa* PAO1 and so precise measurement of intrinsic fluorescence is essential for the accurate detection of gene expression using chromosomal *gfp* fusions.

5.6.2 Stability of Gfpmut3 protein

The *gfp*mut3 mutant of GFP was initially chosen for this study because a wide selection of fusions is available and the mature protein demonstrates increased fluorescence compared to the wild type (Cormack *et al.* 1996). However, the mature protein has a long half-life within the cell, with significant levels detected after 24 hours (Tombolini *et al.* 1997). This was supported by the current study which showed log-phase starter cultures inoculated into fresh broth produced high levels of fluorescence, comparable to those achieved in stationary phase culture. Although this is regarded as a positive feature for qualitative methods such as fluorescence microscopy, it does mean that only upregulation of gene expression can be monitored using this direct measurement technique and any slowing in the rate of expression or even repression cannot be detected. However, this could be achieved using unstable variants of *gfp* which are susceptible to denaturation by host cell proteases, as they possess a comparable fluorescence to more stable gfp-fusions but demonstrate a half-life of less than 15 minutes (Andersen *et al.* 1998). This would allow the monitoring of transient gene expression in planktonic and biofilm culture.

5.6.3 Detection of gene expression using *rpoS*::*gfp*mut3 plasmid fusions

Results achieved using plasmid-borne *rpoS*::*gfp*mut3 fusions showed that fluorescence increase proportionally to cell number in cultures grown under all nutrient limitations which suggested that the *rpoS* signal was being overexpressed. Very high fluorescence values generated by the fusion strains made it difficult to

monitor any subtle increases in expression through the growth cycle. This is unsurprising when considering that pSS429 (*rpoS*::*gfp*mut3 plasmid) is constructed using the *E. coli-Pseudomonas* shuttle vector pUCP19 which is expressed in *P. aeruginosa* PAO1 between 10 and 25 copies per cell (145), which would correlate to at least 1 x 10^{11} copies of the plasmid per ml of culture at densities that cells would routinely enter stationary phase in nutrient-replete conditions.

Detection of GFP by fluorescence measurement has previously been compared with *lacZ* reporters and has been shown to be of equal sensitivity in both qualitative and quantitative assays (Lissemore *et al.* 2000). However, direct measurement of GFP has distinct advantages over other techniques as it does not require specific growth media or a cell lysis step, which facilitates the non-invasive monitoring of gene expression in living tissues (Naylor 1999). However, the use of high copy number plasmids remains a contentious issue especially regarding how accurately they reflect gene expression. GFP may pose a metabolic burden on the recombinant bacterium and can even be toxic when expressed at high levels (Lehtinen *et al.* 2003), which may alter gene expression and consequently the variable of interest. Furthermore, variations in plasmid copy numbers between individual cells and GFP-dependent toxicity resulting in plasmid loss (Wendland and Bumann 2002) suggests potential variability in expression levels between cells. The cellular half-life of GFPmut3 is in excess of 24 hours (Cormack *et al.* 1996) which results in the accumulation of large amounts of GFP protein under conditions of high expression. However, if high levels of fluorescence are required in conditions of low sensitivity or for the investigation of rare mRNA transcripts, high copy number plasmids could be used in conjunction with unstable variants of GFP (Andersen *et al.* 1998). The rapid degradation of GFP within the cell by host proteases would ensure the protein would be maintained at levels that would not significantly influence cellular processes and the downregulation of gene expression could also be monitored, which is unachievable using stable forms of GFP.

Although some studies have approached effects of nutrient starvation on *rpoS* mutants in *P. aeruginosa* (Suh *et al.* 1999), (Jorgensen *et al.* 1999), no study has directly quantified gene expression under these conditions and so conditions in which *rpoS* expression is repressed have not been determined. However, growth of the plasmid-borne fusion strain in conditions that have been confirmed to repress *rpoS* expression would determine if the signal is being overexpressed or at least not accurately representing the expression of *rpoS*. Other studies using plasmid-borne *lacZ* fusions have used a strain containing a copy of the parental plasmid which contains the reporter sequence with no promoter (Xu *et al.* 2001) and although SS225 (*P. aeruginosa* containing blank pUCP20 plasmid) was used as a negative control in this study, the use of a pUCP20 plasmid containing *gfp*mut3 fused to a scrambled promoter would be a more representative control for non-specific expression.

Overall, results suggested that plasmid-borne *rpoS*::*gfp*mut3 were not accurately reflecting actual expression of *rpoS* within the cell. The use of single copy number fusions has been shown to accurately measure individual gene expression *in vitro* and during infection of mammalian cells (Hautefort *et al.* 2003) and so results obtained using a chromosomal *rpoS*::*gfp*mut3 fusion are probably more representative of cellular events.

5.6.4 *rpoS* expression in nutrient-replete medium

Planktonic cultures of *P. aeruginosa* grown in CDM_{10} complete at 37°C routinely reach a final cell density of at least 7 $x10^9$ cells/ml which is less than one generation before the theoretical maximum density of $1x10^{10}$ cells/ml achievable in this medium. Nutrients are added to CDM_{10} complete in excess; however cells can sense and respond to approaching nutrient limitation at least two generations before entry into stationary phase (Brown and Gilbert 1995). Consequently, when cells are grown in nutrient replete medium it is difficult to determine whether entry into stationary phase was induced by cells approaching limitation of a particular nutrient or multiple nutrient stresses including oxygen limitation

A similar *rpoS* expression pattern was noted in both plasmid and chromosomal fusions grown in CDM_{10} complete with levels increasing from inoculation and peaking in stationary phase at levels higher than that achieved under glucose, magnesium and iron limitation. Oxygen limitation has been shown not to affect *rpoS* expression in *P. aeruginosa* biofilms (Xu *et al.* 2001) but has not been fully addressed in planktonic culture. As biofilm and planktonic cells can differ markedly in phenotype (almost 800 proteins showing increased induction in biofilm compared to planktonic culture (Sauer *et al.* 2002)) it would be difficult to correlate the effects of oxygen limitation on *rpoS* expression in biofilm culture with expression in planktonic culture.

5.6.5 *rpoS* expression in carbon-limited medium

Carbon limitation has been shown to increase *rpoS* expression in planktonic culture in *E. coli* (Lange and Hengge-Aronis 1991) but due to the potential iron-restricted phenotype induced by growth in M9 minimal medium, the induction has been suggested to be in response to iron limitation. The experiments have been repeated in an iron-replete medium and have detected no *rpoS* expression in response to glucose limitation (McAuliffe 2002). *P. aeruginosa rpoS* mutants have been shown to be 8 to 12-fold more sensitive to carbon starvation than the parental strain (Suh *et al.* 1999), (Jorgensen *et al.* 1999) which is considerably lower than the 50-fold sensitivity noted in *E. coli* (Lange and Hengge-Aronis 1991). However, RpoS does not appear to confer any increased resistance to carbon starvation when *P. aeruginosa* was grown in succinate minimal medium or LB broth (Suh *et al.* 1999) suggesting that in *P. aeruginosa*, *rpoS* expression is only upregulated in response to limiting concentrations of certain carbon sources.

Opposing results were achieved using plasmid and chromosomal *rpoS*::*gfp*mut3 fusions in response to carbon-limitation. The plasmid fusion indicated a sharp increase in *rpoS* from the point of inoculation reaching a peak at the onset of stationary phase whilst the chromosomal fusion demonstrated no increase in expression through the growth cycle. The latter result does correspond to recent studies showing no expression of *rpoS* under glucose limitation (McAuliffe 2002)

whilst results achieved using the plasmid fusion may be an exaggeration of the actual expression of *rpoS*. The long half-life of GFP coupled with high expression of the fusion throughout the growth cycle means that even under conditions of low expression (such as glucose limitation) subtle changes cannot be detected due to the overexpression of *gfp* from the plasmid fusion. This was not a problem with the chromosomal fusions, which fluoresce at much lower levels; ensuring subtle changes in expression can be detected.

5.6.6 *rpoS* expression in magnesium-limited medium

Magnesium is an essential cofactor in many biochemical reactions and magnesium starvation has been shown to increase bacterial virulence in gram-negative by increasing LPS levels (Guina et al. 2003) and increasing resistance to antimicrobials such as polymyxin B (Wright and Gilbert 1987). This study showed that in both plasmid and chromosomal fusions grown in magnesium-limited conditions, *rpoS* expression was upregulated on entry into stationary phase, after which levels continued to increase. A study by Guina *et al* (Guina *et al.* 2003b) has defined 145 proteins either induced or repressed in response to magnesium starvation including a 3-fold increase in the quorum sensing regulator *lasI*. This gene is responsible for the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone which activates the transcriptional regulator LasR (Pearson *et al.* 1994) which has been shown to regulate *rpoS* expression in *P. aeruginosa* (Latifi *et al.* 1996). Furthermore, *P. aeruginosa* possesses a third QS system, termed the quinolone signalling pathway which involves a 2-heptyl-3-hydroxy-4-quinolone autoinducer termed *Pseudomonas* quinolone signal (PQS) (Pesci *et al.* 1999). PQS has been suggested to act as an intermediate between the *las* and *rhl* QS systems (Mcknight *et al.* 2000) and its levels have been shown to increase five-fold in response to magnesium limitation. This study and previous research suggests that increases in *P. aeruginosa rpoS* expression in magnesium-limited culture is in part, regulated through stimulation of QS systems. However direct measurement of RpoS levels in these conditions would have to be undertaken to fully confirm this hypothesis.

5.6.7 *rpoS* expression in iron-limited medium

Both the chromosomal and plasmid *rpoS*::*gfp*mut3 fusions demonstrated increased expression in response to iron-limitation. Expression in the chromosomal fusion began to increase at least two generations before stationary phase suggesting that iron depletion is detected before any reduction in growth rate is noted and gene expression is altered accordingly, which is in agreement with previous research (Lodge *et al.* 1986). This appeared not to be the case with the plasmid fusion grown in iron-limited medium, whereby *rpoS* expression was upregulated only as cells entered stationary phase. Results using both fusions were compromised by high background fluorescence (represented by the wild type strain). Pyoverdine is normally produced as cells enter stationary phase but levels are largely elevated in response to iron limitation (Liu and Shokrani 1978). Although the fluorescence generated by chromosomal *rpoS*::*gfp*mut3 fusions is comparatively low it still remains higher than the intrinsic fluorescence of *P. aeruginosa.* However, if intrinsic fluorescence is increased by elevated pyoverdine, the fluorescence of the fusion strains appears no higher than background levels. This is consolidated by iron-limited wild type cultures producing higher fluorescence values than the corresponding cultures grown in nutrient-replete, magnesium-limited and glucose-limited conditions.

Null-mutants of *rpoS* have been shown express increased levels of pyoverdine and pyocyanin and reduced levels of exotoxin A (Suh *et al.* 1999), (Whiteley *et al.* 2000), (Vasil and Ochsner 1999). This suggests that *rpoS* is intricately involved in iron-dependent gene expression, most probably through the alternative sigma factor PvdS which regulates production of both pyoverdine (Cunliffe *et al.* 1995) and exotoxin A (Ochsner *et al.* 1996). Furthermore, iron starvation has also been shown to cause significant increases in *lasI* transcription (Bollinger *et al.* 2001) and as lasR has been shown to regulate *rpoS* expression (Latifi *et al.* 1996) the response of *P. aeruginosa* to iron limitation must at least be partly controlled by QS. The increased intrinsic fluorescence of wild type cultures in iron-limited conditions in this study correlate with elevated levels of pyoverdine and pyocyanin reported to occur in response to iron starvation (Vasil and Ochsner 1999). Studies have also indicated that pyoverdine and pyocyanin expression is repressed by RpoS (Suh *et al.* 1999) and so

the elevated intrinsic fluorescence of wild type cultures may be the result of an iron-dependent repression of *rpoS*. This correlates with the minimal levels expression obtained using chromosomal *rpoS*::*gfp*mut3 fusions.

5.6.8 *rpoS* expression in biofilm culture

Relatively few studies have investigated the expression of *rpoS* in biofilm culture; however *rpoS* expression in continuously-fed *P.aeruginosa* biofilms has been shown to exceed levels in planktonic stationary phase cultures (Xu *et al.* 2001). This contradicts another study using microarray technology which has shown a 2-fold reduction in *rpoS* expression after 10 days of biofilm growth (Whiteley *et al.* 2001). The discrepancies in these results can be explained by the biofilms assayed in the latter study being over 3 times older than those sampled in the former study and as *lasI* expression is reported to decrease after the initial attachment stages of biofilm formation (De Kievet *et al.* 2001) the reduction in QS regulation in older biofilms correlates with the reported decrease in *rpoS* expression. The *las/rhl* quorum sensing systems of *P. aeruginosa* are reported to regulate *rpoS* expression (Latifi *et al.* 1996), (Greenberg 1998) and QS has been reported to play a role in biofilm growth, with high *lasI* expression noted in initial stages of biofilm attachment after which levels reduced as the biofilm developed (De Kievet *et al.* 2001) Furthermore, whilst *lasI* and *rhlI* mutants grown in glucose minimal medium produce sparse, thinly populated biofilms, with normal structures being restored with the addition of exogenous AI. However, the addition of exogenous AI cannot restore the wild type phenotype to cells grown in a citrate minimal medium, suggesting that QS–dependent formation of the biofilm can be carbon source dependent. This corresponds with studies that proposed *rpoS* resistance to starvation in planktonic culture is also carbon source dependent (Suh *et al.* 1999). Consequently, the involvement of QS and RpoS in the biofilm phenotype, although important in the initial stages of development, appears to be transient and largely environmentally dependent.

5.6.9 Detection of biofilm gene expression using *rpoS*::*gfp*mut3 chromosomal fusions

The Bühler method of biofilm culture was used in this study so that specific nutrient limitation could be achieved to investigate the expression of *rpoS* in biofilm culture (Buhler *et al.* 1998). The sessile communities produced using this method may not fit the classical description of a biofilm, which is a microbial consortium at a phase interface such as air-liquid phase or solid-liquid phase (Jenkinson and Lappin-Scott 2001), but the technique is useful to compare gene expression in biofilm and planktonic cultures grown under the same nutrient-limited conditions.

As results suggested that the plasmid was overexpressing the *rpoS* signal, it was decided to use the chromosomal fusion *rpoS*::*gfp*mut3 fusion in the biofilm study as it would be more reflective of the cellular levels of *rpoS* expression. Small increases in fluorescence were noted in *P. aeruginosa* biofilms grown in nutrient replete conditions but biofilms grown under carbon, magnesium and iron limitation showed minimal expression of *rpoS*. Even though the expression of *rpoS* in biofilms is reported to differ to that of planktonic growth (Xu *et al.* 2001) results indicated that the method used in this study was not sufficiently sensitive to detect fluorescence produced by the *rpoS*::*gfp*mut3 chromosomal fusions.

5.6.10 Alternative methods for measuring gene expression in *P.aeruginosa* biofilms

There are alternative techniques that also incorporate GFP which could be used to *rpoS* expression in *P. aeruginosa* biofilms. The biofilm growth method of O'Toole and Kolter (O'Toole and Kolter 1998b) could be easily applied the system used in this study. *P. aeruginosa* biofilms would be grown in 96-well plates and fluorescence could be monitored through the growth cycle using a 96-well spectrofluorimeter. This would increase the number of samples that could be processed and furthermore, growth would be at the solid/liquid interface and so can be accurately defined as biofilm growth. The majority of techniques used to measure gene expression in biofilms, such as microarray technology (Whiteley *et al.* 2001) and *lacZ* reporters (Xu *et al.* 2001) monitor the population as a whole. However, a biofilm comprises of a

heterogeneous population cells (Costerton *et al.* 1999) which demonstrate different phenotypes depending on their location within the larger structure (Davies *et al.* 1998). Therefore more cell-specific techniques are required to investigate the *in situ* expression of genes within the biofilm. This can be achieved using a combination of flow cells, confocal scanning laser microscopy and COMSTAT image analysis (Klausen *et al.* 2003) and as this technique is not invasive, temporal expression of genes within the biofilm can be easily monitored.

6 CONCLUDING REMARKS

6.1 COMMENTS AND PERSPECTIVE

6.1.1 The role of SMART in future studies

Currently quantitative PCR techniques are the most sensitive methods for monitoring gene expression; other techniques with lower sensitivity are usually overlooked. However, PCR-based methods do have their shortcomings, being prone to contamination by nucleic acids and a wide selection of common compounds can inhibit amplification and produce variable results (Wilson 1997). Nevertheless RT-PCR sometimes remains the only option when quantifying targets at very low concentrations. However, in situations that require the detection of a target at higher concentrations, other techniques such as the SMART assay can be used. It is a simple yet reproducible technique that is not affected by the problems that disrupt PCR, such as variable amplification levels and false positive results caused by target contamination. This study showed that even simple modifications in experimental design could significantly increase assay sensitivity and so further assay optimisation would make the SMART assay an even more competitive technique for measuring gene expression. Negative aspects aside, plasmid fusions remain widely used in the quantification of gene expression and this study has shown that the SMART assay can reproducibly detect gene expression from high copy number plasmid-borne fusions. Consequently, low copy number fusions may be an ideal choice for future research as they would be expressed at levels that are detectable by the SMART assay but the lower levels of GFP expression would have a less detrimental effect on cell metabolism.

6.1.2 Overexpression of GFP

Although non-toxic at low concentrations, GFP can be toxic in conditions of high expression (Lehtinen *et al.* 2003) and this study has shown that the high levels of fluorescence generated by overexpression of *gfp*mut3 from high copy number plasmids can also mask subtle changes in gene expression. When considering the widespread use of plasmid-borne *gfp* fusions it is surprising that there has been very

little investigation of the effects of GFP on bacterial cell metabolism. As no readily identifiable markers of toxicity, such as reduction in growth rate and growth yield are usually noted in cells expressing GFP, it is assumed that the high levels of protein do not have a detrimental effect on cellular metabolism. However, it remains unclear as to what extent plasmid-borne *gfp* fusions actually reflect the expression of the gene. The popularity of plasmid fusions can be explained by certain advantages compared with chromosomal fusions. Firstly, large amounts of signal can be generated from gene fusions under the control of a strong promoter or when expressed from a high copy number plasmid, which can increase detection sensitivity at low cell numbers. Secondly, by using shuttle vectors and other cloning tools the construction of *gfp* fusions is relatively simple and they can be easily transformed into different strains and species. Conversely, the insertion of a gene fusion into the chromosome of an organism is more difficult to achieve but as chromosomal fusion proteins are expressed at far lower copy numbers than most plasmid-borne fusions they are thought to be more reflective of cellular expression levels. Therefore, further studies are required to determine how closely plasmid fusions (especially high copy number) reflect actual gene expression and also to determine to what extent high expression of *gfp* fusions are detrimental to the cell.

6.1.3 The use of complex media in gene expression studies

Genome sequencing and the subsequent advent of microarray technology has enabled the study of global gene expression in a variety of organisms in a far shorter time than would achievable using other techniques. It has revealed how an organism's genome responds to environmental stimuli and the use of knockout mutants has revealed interactions and levels of regulation which would not have been determined by monitoring the expression of single genes. However this technology does have its limitations, but they are not exclusive and can be equally applied to other gene expression techniques such as RT-PCR and gene fusion technology. These shortcomings are typified by a study by Oschner *et al* (Oschner *et al.* 2002) which used microarray technology to investigate the effects of iron-limitation on global gene expression in *P. aeruginosa.* Bacteria were grown in Tryptone-Soya broth (TSB), in iron-limited and iron-replete conditions and then a microarray was used to compare

genomic expression. Bacteria grown in complex media such as TSB enter stationary phase in response to undetermined stress which is commonly assumed to be oxygen limitation. However, as the concentrations of the medium components are undefined and so entry into stationary phase could be in response to a combination of oxygen limitation and specific nutrient starvation. Therefore, the aforementioned study was actually comparing the gene expression of iron–limited cells with cells under unspecified stress(es). Iron-starved and iron-replete cells were sampled at densities of 4×10^9 cfu/ml and 6×10^9 cfu/ml respectively. This indicated that iron starved cells were entering stationary phase less than one generation before cells grown iron-replete conditions. Bacteria can sense approaching nutrient starvation and adjust gene expression without any reduction in growth rate at least two generations before entry into stationary phase (Lodge *et al.* 1986). As the iron limited cultures entered stationary phase at a final density so close to iron-replete conditions, the variations in global gene expression detected using the microarray were probably the result of multiple stresses. This could be a combination of iron-limitation and undefined stresses that induce growth cessation in iron-replete conditions.

This problem is apparent in other studies using microarray technology; Whitely *et al* (Whitely *et al.* 2001) used a defined medium to investigate global gene expression in the biofilm culture and a combination of the ribonuclease A assay and *lacZ* fusions were used a control for measuring gene expression. However, the cultures used in the control reactions were grown in another undefined medium, LB broth. Consequently, the results of the microarray analysis were being validated against results obtained using cells grown in a different environment which may demonstrate an altered pattern of expression.

These problems are not exclusive to microarray studies, results achieved using *lacZ* fusions are often misinterpreted due to unsuitable growth conditions and differing experimental design as illustrated by the conflicting data regarding the position of *rpoS* in the QS hierarchy (Latifi *et al.* 1996) and (Whitely et al. 2000). When bacteria are grown in complex media such as LB and TSB entry into stationary phase is a response to undefined stresses with which gene expression is altered accordingly

making it impossible to compare data between gene expression studies if they have been conducted using different complex media. This problem can be resolved by using a chemically defined medium as this would allow accurate comparison between studies and ensure that results are not influenced by an unspecified stress response.

6.1.4 The importance of an iron-replete phenotype

Not all problems are solved simply by using a defined medium, as this study showed that cells grown in CDM_{10} containing 10 μM iron were in fact limiting for iron due to MOPSO buffer reducing iron availability. MOPS buffer is used as a replacement for phosphate buffer when studying the effects of phosphate limitation and has been used in numerous studies including the effects of phosphate and glucose limitation on *rpoS* expression (Jorgensen *et al.* 1999), protein expression in response to phosphate starvation (Madhusudhan et al. 2003) and the activation of QS by the stringent response (Van Delden *et al.* 2001). All of these studies used a MOPS-buffered minimal medium to grow *P. aeruginosa* cultures under specific nutrient limitations. Therefore, the results obtained using these media were influenced by multiple stresses including iron limitation, as opposed to a response to a single starvation. Since other studies have revealed that another commonly used buffer, HEPES can reduce iron availability in solution (Tadolini 1987), it is imperative that an iron-replete phenotype be confirmed in a growth medium before any experiments are undertaken, especially when investigating organisms with a relatively high iron requirement such as *P. aeruginosa*. This study has shown that an iron-replete phenotype can be confirmed easily using a combination of SDS-PAGE and pyoverdine quantification. However, these techniques are not applicable to organisms that do not produce siderophores but as iron-regulated gene expression is well characterised in many organisms, RT-PCR can be used as a suitable alternative by detecting the expression of iron-repressed genes. Consequently, this would confirm the iron status cultures grown in any given medium.

6.1.5 Non-invasive measurement of gene expression

Many techniques used to monitor gene expression require the isolation of total RNA from cells. Methods used to extract RNA often involve multiple processing steps which including cell lysis by lysozyme and sonication, the use of toxic reagents such as phenol:chloroform and mercaptoethanol, in conjunction with multiple centrifugation and precipitation steps (Sambrooke *et al.* 1989). All of these can impose physical or chemical stresses upon the cell which may alter the gene expression. This becomes especially important when investigating the expression of genes such as *rpoS* that are directly involved in the bacterial stress response whereby bacteria may respond to the stress of sample processing as opposed to the intended biological stimulus. The use of *gfp* fusions to monitor gene expression can provide more information compared to other techniques, combining non-invasive quantification of gene expression by fluorescence measurement with localisation of gene expression using scanning laser confocal microscopy (Silhavy 2000) and non-invasive measurement of gene expression can not only eliminate the potential inaccuracies incurred by sample processing but it can also be used to monitor gene expression in"real time".

Fusion technology does provide the means to study gene expression in a manner previously unachievable using other techniques and this study has shown that *gfp* expression can be measured in two very different ways; either by direct measurement of GFP fluorescence or quantification of *gfp* mRNA by the SMART assay. Every technique has both its benefits and its shortcomings and although these methods can provide a wealth of information on an individual basis, the scope of any study can be widened significantly by using a combination of technologies. Therefore, through techniques such as microarray, quantitative PCR and gene fusions, bacterial gene expression can be investigated in far greater detail than using one method alone. However, this can only be achieved if bacteria are grown in a truly defined medium which provides sufficient iron to support growth as varying growth conditions make it very difficult to accurately compare studies that have used the same technique. These problems can be even more pronounced when comparing different techniques that have used a variety of growth conditions.

Therefore, any conclusions regarding gene expression in a bacteria can be only accurately compared between studies when it has be ensured that bacteria have been grown in comparable environmental conditions.

6.2 SUGGESTIONS FOR FURTHER WORK

1. Investigation of the effects of cycled amplification on the sensitivity of the SMART assay

2. Investigation of the effects of nutrient limitation on *rpoS* expression in *P. aeruginosa* biofilms using direct fluorescence measurement but in a 96-well format

3. Further clarification of the effects of MOPSO on the availability of iron in biological media.

4. Further investigation into the expression of *rpoS* under starvation conditions using a combination of RT-PCR and reporter fusions

5. An accurate comparison of the effects of plasmid-borne and chromosomal *gfp* fusions in the cell and to determine how accurately they reflect gene expression

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APPENDIX 1.1 SMART ASSAY PROBE SEQUENCES

Probe	Function	Sequence
Ampi 1491	Extension probe for *P. aeruginosa* 23S rRNA	GTTACTCTTTAGGAGGAGACCGC CCAGTCTTCGAAAT
Ampi 1492	Template probe for *P.aeruginosa* 23S rRNA	TCGTCTTCCGGTCTCTCCTCTCA AGCCTCAGCGCTCTCTCTCCCTA TAGTGAGTCGTATTAATTTCGAA AAACTGCCCACCATACACTGACC TCGATCC
Ampi 1493	Target probe containing *P. aeruginosa* 23S rRNA target sequence	GCTTGAGGTTCTAACTCTGGTCC GTAATCCGGATCGAGGACAGTGT ATGGTGGGCAGTTTGACTGGGGC GGTCTCCTCCTAAAGAGTAACGG AGGAGTACGAAGGTGCGCTCAGA CCGGT
Ampi 1587	Extension probe for *gfp*mut3	TTTACCGTAAGTAGCATCACCTT CACCTTCTTCGAAAT
Ampi 1588	Template probe for *gfp*mut3	TCGTCTTCCGGTCTCTCCTCTCA AGCCTCAGCGCTCTCTCTCCCTA TAGTGAGTCGTATTAATTTCGAA ACCGGAGACAGAAAATTTGTGAC CATTAAC
Ampi 1586	Target probe containing *gfp*mut3 target sequence	GTCCCAATTTTGGTTGAATTAGA TGGTGATGTTAATGGTCACAAAT TTTCTGTCTCCGGTGAAGGTGAA GGTGATGCTACTTACGGTAAATT GACCTTAAAATTTATTTGTACTA CTGGT
Ampi 859	Amplification probe	TGCCTGCTTGTCTGCGTTCTGGA TATCACCCGAGTTCTCGCTTCCT ATAGTGAGTCGTATTAATTTCTC GTCTTCCOGGTCTCTCCTCTCAA GCCTCAGCGCTCTCTCTCCC
Ampi 1117	Additional amplification probe	GGTGGCTCGGCTCGCGTGCGTGA TCGATGGTTGTCTCTTCCTATAG TGAGTCGTATTAATTTCTGCCTG CTTOGTCTGCGTTCTGGATATCA CCCGAGTTCTCGCTTCC
Ampi 111	Complement probe	ATATCACTCAGCATATTAAAGCT T
Ampi 1679	Extension probe facilitator	GTAGTACAAATAAATTTTAAGGT CAA
Ampi 1680	Template probe facilitator	ATCACCATCTAATTCAACCAAAA TT

APPENDIX 1.2 ELOSA PROBE SEQUENCES

Probe	Function	Sequence
Ampi 506	Capture probe for 3WJ step	TCGTCTTCCGGTCTCTCCTC
Ampi 507	Standard curve probe for 3WJ step	TCGTCTTCCGGTCTCTCCTCTCA AG
RNA3-AP	Detection probe for 3WJ step	AGCAGAAGGCAAGAGAGGAGAGT TCGGAGTCGCGAGAGAGAGGGAT ATCACTCAGCATAAT
Ampi 542	Capture probe for amplification step	TCTGCTGCCTGCTTGTCTGCGTT CT
Ampi 543	Standard curve probe for amplification step	GGGACTGACGATTCGGGTGATAT CCAGAACGCAGACAAGCAGGCA
Wallac-AP	Detection probe for amplification step	GGATATCACCCG
Ampi 895	Capture probe for additional amplification step	TCGTGTCTGGTGGCTCGGCTCGC GT
Ampi 896	Standard curve probe for additional amplification step	GGAAGCGAGAACCATCGATCACG CACGCGAGCCGAGCCACCAGA
New Wallac-AP	Detection probe for for additional amplification step	ACGGACGAACAGACGCAAGACCT ATAGTGGGCTCAAGAGCGAAGG

APPENDIX 2.1 DETERMINATION OF OPTIMAL WAVELENGTH FOR MEASUREMENT OF OPTICAL DENSITY

Experiments were undertaken to determine if cell cultures and cell-free supernatants produced absorbent compounds which could influence OD measurement. Cultures of *P. aeruginosa* PAO1 were incubated for 16 h at 37°C in either LB broth or CDM_{10} complete. Cultures were then diluted 1:200 in the appropriate medium and aliquots were centrifuged at 10,000 *g* for 5 min. The absorbance of both the culture and the culture supernatant were measured at wavelengths from 400 to 600 nm.

Cultures grown in CDM_{10} displayed a gradual reduction in absorbance with increasing wavelength and the cell-free supernatants displayed no absorbance at investigated wavelengths (Fig. 8.1). Cultures grown in LB broth revealed an initial increase in absorbance at wavelengths from 400 to 440 nm, followed by a gradual decrease at higher wavelengths (Fig. 8.2). These results were mirrored by the culture supernatant samples with absorbance peaking at 440 nm then steadily decreasing at higher wavelengths. A wavelength of 470 nm was selected for measurement of optical density in this study as it provided similar levels of discrimination between the absorbance of the cultures and the cell-free supernatant in both CDM_{10} complete and LB broth.

Fig. 8.1 Screen for absorbent cell excretions of *P. aeruginosa* PAO1 grown in CDM_{10} complete. The absorbance of *P. aeruginosa* PAO1 cultures grown at 37°C in CDM_{10} complete (•) and the corresponding cell-free supernatant (○).

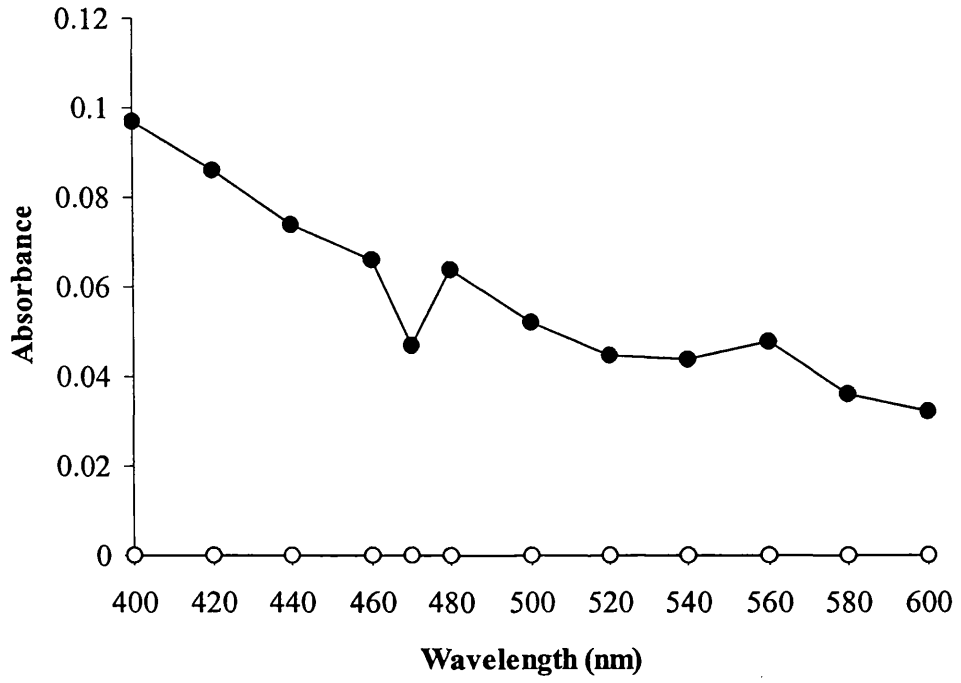
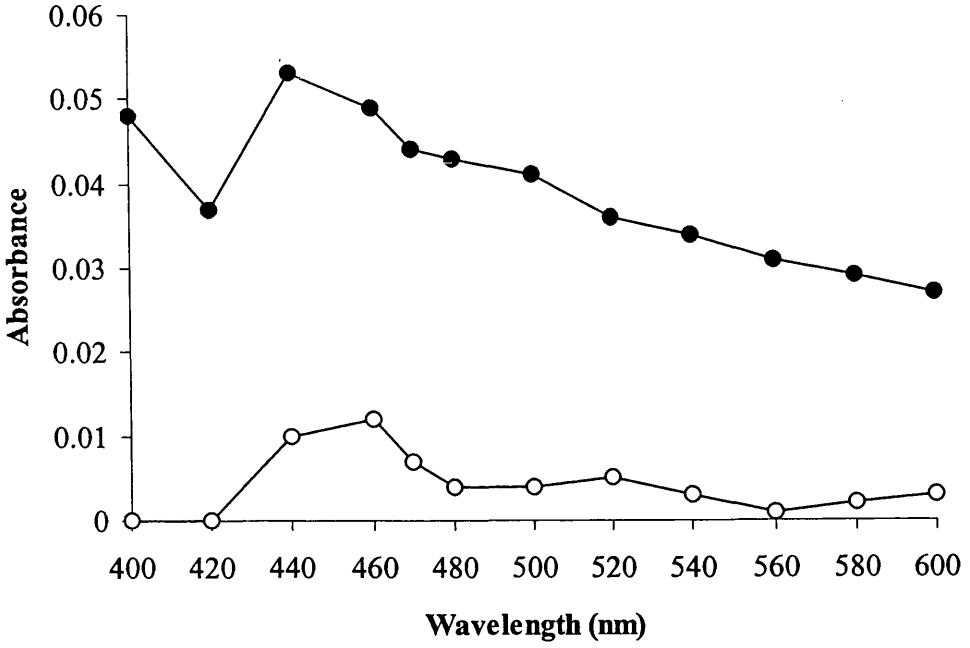


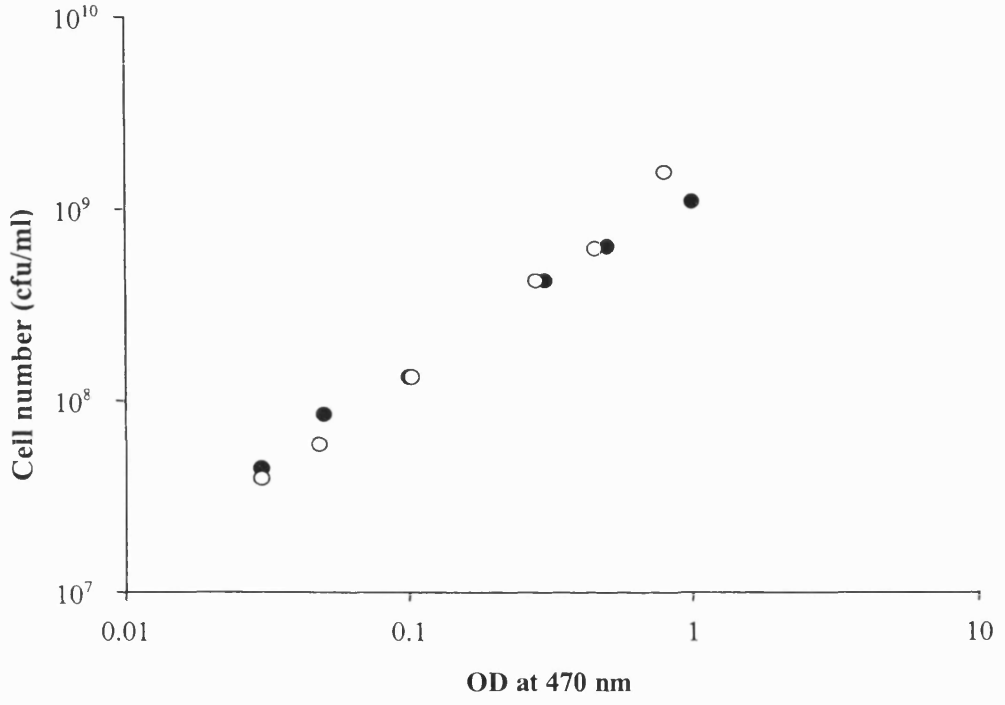
Fig. 8.2 Screen for absorbent cell excretions of *P. aeruginosa* PAO1 grown in LB broth. The absorbance of *P. aeruginosa* PAO1 cultures grown at 37°C in LB broth (●) and the corresponding cell-free supernatant (○).



APPENDIX 2.2 DETERMINATION OF THE RELATIONSHIP BETWEEN COLONY COUNT AND OPTICAL DENSITY

Cultures of *P. aeruginosa* PAO1 were incubated in LB broth for 6 h at 37°C. Starter cultures were produced by re-suspending cells to an OD of 0.002 in pre-warmed CDM_{10} complete which were then incubated for 16 h at 37°C. Cells were re-suspended in 20 ml of pre-warmed CDM_{10} complete and then incubated at 37°C until reaching log phase (OD 0.9) and stationary phase (OD 9). Cultures were then serially diluted in 0.9% saline and OD readings were taken at 470 nm. Concurrently, sample colony counts were determined in triplicate using the Miles-Misra drop plate method (Miles et al, 1938). Briefly, cultures were diluted appropriately in CDM_{10} complete and 10 µl volumes were dropped, in triplicate, onto overdried LB-agar plates which were incubated at 37°C for 16 h. Colonies were counted and cell density was calculated as colony forming units per ml (cfu/ml). Results shown in Fig. 8.3 indicate that in both log and stationary-phase cultures, an OD of 1 corresponded to an approximate cell density 1×10^9 cfu/ml.

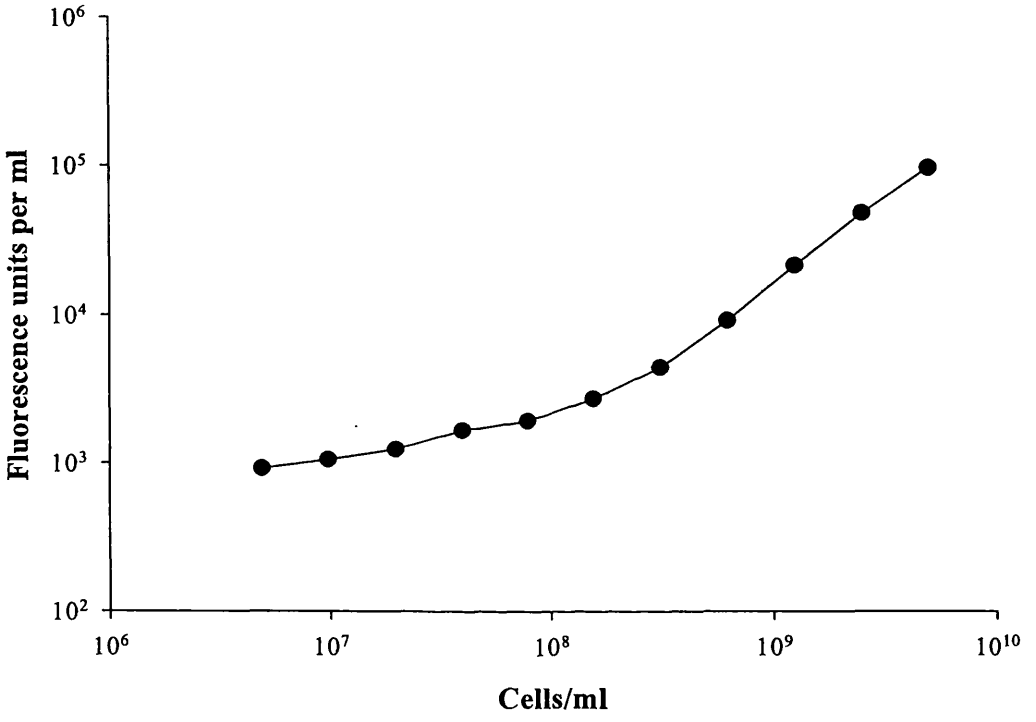
Fig 8.3 The effect of growth phase on the relationship between optical density at cell number in *P. aeruginosa* PAO1 grown in CDM_{10} complete. Optical density at 470 nm as a function of cell number in log phase (●) and stationary phase (○) cultures of *P. aeruginosa* PAO1 grown in CDM_{10} complete.



APPENDIX 2.3 MINIMUM LEVEL OF DETECTION OF FUSION STRAIN FLUORESCENCE IN PLANKTONIC CULTURE

The *P. aeruginosa rpoS::gfp*mut3 chromosomal fusion SS336 was selected to determine the minimum sensitivity of fluorescence measurement of the *gfp* fusions used in this study as it would provide the lowest peak fluorescence signal. Cultures were grown to stationary phase in CDM_{10} complete then serially diluted 1:1 in 0.9% saline. Cell number was calculated from OD at 470 nm (assuming an OD of 1 corresponded to approximately 1 x 10^9 cells/ml) and culture fluorescence was measured using a Photon Technology fluorimeter at 480 nm excitation and 511 nm emission. Fig. 8.4 illustrates a linear relationship between OD and fluorescence from 1 x 10^5 fu/ml down to approximately 7 x 10^3 fu/ml. Below this value the relationship was not linear and therefore fluorescence readings outside this range would not be considered reproducible.

Fig. 8.4 Determination of minimum sensitivity of fluorescence measurement of *P. aeruginosa* PAO1 *gfp* fusions in planktonic culture. Fluorescence of stationary phase cultures of *P. aeruginosa* SS336 serially diluted 1:1 in saline; OD_{470} and fluorescence (480 nm emission/ 511 nm excitation) were measured for each dilution. n=3.



APPENDIX 2.4 THE EFFECTS OF SAMPLE PROCESSING ON *RPOS* EXPRESSION IN BIOFILM CULTURE

Biofilms were grown using a reproducible nutrient-depleted method (Buhler *et al.* 1998) which involves propagating biofilms at 37°C on nitrocellulose membranes placed on CDM agar. To determine cell numbers and fluorescence from biofilms, the membrane is removed from the agar, placed in a 30 ml sterile polystyrene universal tube containing 5 ml of 0.9% saline then vortexed for 30 seconds. An assay was undertaken to ensure that the dispersion of biofilm cells did not upregulate *rpoS* expression.

Biofilms were harvested after incubation at 37°C for 14 h on CDM_{10} complete agar. This provided cells in which *rpoS* expression was at a minimum in nutrient-replete conditions but at a density sufficient to detect any increase in fluorescence. Fig. 8.5 illustrates that the fluorescence of *P. aeruginosa* SS336 did not increase above background levels this was supported by an unpaired t-test which confirmed no significant difference between the fluorescence of *P. aeruginosa* SS336 and *P.aeruginosa* PAO1, confirming that *rpoS* expression was not upregulated by the processing steps of the assay.

Fig. 8.5 Determination of the effect of experimental processing on the expression of *rpoS* in *P.aeruginosa* PAO1 biofilms. The fluorescence of *P. aeruginosa* PAO1 wild type (black bar) and the chromosomal *rpoS*::*gfp*mut3 fusion *P. aeruginosa* SS336 (white bars) incubated for 14 h at 37°C on CDM complete agar. n=3, ± SEM.